CHOLESTANOL IN AVIAN PLUMAGE

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SUMMARY.

Considerable amounts of cholestanol were consistently found in the feather lipids obtained by ether or chloroform extraction of the plumage of 14 (widely varying) species of birds including one ratite (emu). The cholestanol, which is the characteristic steroid of avian plumage, occurred mainly as the stearate and as the free steroid.

Compared with those of other birds, the feathers of the flightless emu contained about half the amount of cholestanol. The feather lipids, which are by-products of keratinisation, waterproof the plumage.

INTRODUCTION.

It has previously been shown (Bolliger and Gross, 1958) that the plumage of several species of birds contains among other lipids a steroid alcohol "plumin steroid" and its stearate "plumin". These compounds were considered to be related to ergostanol. Subsequently, this assumption became untenable (Bolliger, 1960) and plumin steroid, its acetate, benzoate and stearate were re-examined. In addition, quantitative determinations were performed on the feather lipids and feather steroids of 13 of the 14 widely differing species of birds examined.

MATERIAL AND METHODS.

The rarer birds were obtained from the Zoological Gardens (Taronga Park) where they had died or had been killed. Quantitative results (Table 2) refer to the total plumage, including the complete quills, of each species of bird investigated. As an alternative to extraction with ether at room temperature (Bolliger and Gross, 1958) the immersed feathers were boiled four times of two hours each in chloroform. This mode of extraction yielded about 5 p.c. more feather lipid than the ether method. The chloroform extracts, however, were strongly discoloured which sometimes complicated the subsequent purification.

The feather lipids were then dissolved in hexane or petrol ether (b.p. 60°-90°, Shell X-60), these being found equally efficient. This solution was passed through a column (40 x 2 cm.) of aluminium oxide (B.D.H. for chromatographic analysis) which was completely eluted with petrol ether. The eluate was evaporated to dryness and then boiled for 15 minutes in about 20 volumes of methanol. The mixture was then cooled in a refrigerator for about an hour. The solids present were filtered off and transferred to 20 volumes of fresh methanol for further boiling. This procedure was repeated four times, 40 volumes

of methanol being used for the last boiling. Finally, the remaining solid was dried, redissolved in petrol ether, the solution passed through an aluminium oxide column (30 x 2 cm., B.D.H., for chromatographic analysis) and eluted with petrol ether. After evaporation of the solute the remaining substance was recrystallised from acetone. The resulting product which usually melted above 90° C. and was considered to be "crude" plumin, was weighed.

Treatment with acetic anhydride and sulphuric acid (Heath-Brown, Heilbron and Jones, 1940) removed substances which gave a positive Liebermann-Burchhard reaction. Further recrystallisations from alcohol or acetone containing some carbon tetrachloride and repeated chromatography finally yielded colourless crystals of plumin, m.p. 97° C., $[\alpha]_D + 11°$ (C, 1·6 in chloroform) $\nu_{max.}^{C Cl_4}$, 2915, 1724, 1465, 1173 cm.$^{-1}$. Found: C, 82, 46; H, 12·52. Calc. for C_{45} H_{82} O_2: C, 82, 50; H, 12·62. Plumin was identical with an authentic specimen of cholestanyl stearate prepared from cholestanol and stearic acid m.p. 98° C., $[\alpha]_D + 11°$ (C, 1·8 in chloroform) (Table 1). Ellis and Gardner (1918) reported for cholestanyl stearate m.p. 100° C., $[\alpha]_D + 18·4°$ (in ether).

The first-mentioned column still contained some feather lipids which were eluted with benzene. After evaporation of the benzene the residue was dissolved in about 40 volumes of boiling acetone. On cooling, sparingly soluble substances, including some plumin, crystallised out and were filtered off. The readily soluble plumin steroid remained in the filtrate which was then evaporated to dryness. The resulting dry crystals were redissolved in petrol ether and rechromatographed using benzene for elution. This procedure was repeated three times before the final product was recrystallised from methanol. The resulting crude plumin steroid, melting at 130°-137° C., was weighed. For further purification this product was treated by the method of Heath-Brown, Heilbron and Jones (1940). The resulting mixture of cholestanol and cholestanyl acetate was treated with boiling acetic anhydride (30 mins.) to effect complete acetylation and was then recrystallised from methanol until it melted at 108° C. On saponification and recrystallisation from methanol colourless crystals were obtained m.p. 140° C., $[\alpha]_D + 24°$ (C, 2 in chloroform).

The length of the side chain of this crystalline material was measured by the method of Cox, High and Jones (1958). It consisted of only eight carbon atoms, not nine as required by ergostanol. Furthermore, plumin steroid was shown to be identical with an authentic specimen of cholestanol with respect to elementary analysis, melting point, infra-red absorption and optical rotation. The acetate and the benzoate prepared from purified plumin steroid were also identical with authentic cholestanyl acetate and cholestanyl benzoate (Table 1).

TABLE 1.

Comparison of plumin steroid and authentic cholestanol.

Name	Plumin steroid		Authentic cholestanol	
	m.p. °C	$[\alpha]_D$	m.p. °C	$[\alpha]_D$
Steroid	140	+24	140–141	+24 (Heath-Brown *et al*, 1940)
Acetate	109	+11	109–110	+11 (Heath-Brown *et al*, 1940)
Benzoate	137*	+18	138–140*	+20 (Wieland *et al*, 1941)
Stearate (Plumin)	97	+11	98	+11 Authors

* Clearing at 156 and 157° C. respectively.

Optical rotatory measurements on the ketone and hemiketal (Djerassi, Mitscher and Mitscher, 1959) derived from the plumin steroid indicated that the former was a 3-oxo-5 α steroid with no substituents at C-2 and C-4 and the infra-red spectrum of the ketone was identical with that of cholestan-3-one.

RESULTS.

Identification.

The value obtained in the present work for the specific optical rotation of plumin steroid was considerably higher than that reported in the previous communication (Bolliger and Gross, 1958), presumably because of the additional purification by the method of Heath-Brown, Heilbron and Jones (1940). This value, and the other properties of plumin steroid examined, agreed with corresponding properties of authentic cholestanol showing that the plumin steroids belong to the cholestanol series and not the ergostanol series as previously suggested.

Determination of cholestanol and cholestanyl esters in plumage.

A method was designed for the isolation and semi-quantitative determination of plumin and plumin steroid present in the feathers of a single bird of moderate size. Plumin was usually isolated after a standardised purification procedure as "crude" cholestanyl stearate. The melting point of the product from certain birds was still as much as 10° C. below that of authentic cholestanyl stearate (m.p. 98° C.) and further purification was, in many cases (e.g. the turkey), tedious. Plumin actually melting at 97° C. was obtained from goose, duck and black swan feathers.

Saponification split plumin from all birds examined into cholestanol (identified *i.a.* as the characteristic acetate and by infra-red spectroscopy) and stearic acid. However, as shown by gas chromatography the stearic acid was sometimes contaminated by as much as 10-30 p.c. palmitic acid. The gas chromatography was performed on the methyl esters of the fatty acids as described by James and Wheatley (1956), the stationary phase used being Apieson M vacuum stopcock grease. The palmitic acid probably was derived from cholestanol palmitate which one could expect to occur in plumin. The presence of this or other closely related esters could explain the depressed melting points of certain plumins and some of the difficulties connected with their purification, an aspect which requires further investigation.

The 14 avian species examined represented a variety of terrestrial and aquatic birds as well as one ratite, the emu (*Dromaeus novae-hollandiae*). With the exception of the emu the plumages investigated contained on an average 2 p.c. feather lipids, corresponding to approximately 0·1 to 0·2 gm. of cholestanyl esters and 0·05 to 0·1 grams of free cholestanol per 100 gm. of feathers. No definite difference between land and water birds could be established. How-

ever, the primitive plumage of the flightless emu, which is morphologically different from that of the other birds examined, yielded only about 0·05 gm. plumin and 0·03 gm. plumin steroid per 100 gm. of feathers. The total feather lipids were correspondingly low — 0·4 gm. p.c. or approximately one-fifth of the average value for other species (Table 2).

TABLE 2.

Total feather lipids and approximate amounts of cholestanyl esters and cholestanol from the plumage of 14 different species of birds expressed as per cent of dry feathers. The cholestanyl esters include cholestanyl stearate and cholestanyl palmitate.

Bird	Feather lipids p.c.	Cholestanyl esters p.c.	Cholestanol p.c.
Black Swan *Chenopis atrata*	2·2	0·2	0·1
Cormorant *Phalacrocorax ater*	1·3	0·1	0·07
Duck *Anas* sp.	2·4	0·2	0·1
Fowl *Gallus* sp.	1·2	0·15	0·07
Goose *Anser* sp.	2·4	0·2	0·1
Griffon vulture *Gyps fulvus*	2·4	0·1	0·1
Peahen *pavo* sp.	+	+	+
Pelican *Pelecanus conspicillatus*	2·0	0·15	0·1
Pigeon *Colomba livia*	1·7	0·2	0·1
Seagull *Larus novae-hollandiae*	2·2	0·15	0·1
Stork *Ciconia* sp.	2·1	0·1	0·07
Turkey *Meleagria* sp.	2·1	0·15	0·07
Wedgetailed eagle *Uroaetus audax*	2·3	0·2	0·1
Emu *Dromaeus novae-hollandiae*	0·4	0·05	0·03

Cholestanol, therefore, seems to be a characteristic constituent of the plumage of the whole class of *Aves* and this steroid has not yet been observed with certainty in the keratinous integument of other vertebrates so far examined, although chromatographic evidence for its existence in rat skin has been provided by Stokes, Hickey and Fish (1958).

DISCUSSION.

It has been known for some time that in various tissues small amounts of cholestanol are frequently associated with cholesterol and for many years it was considered to be a metabolite of the latter, but recently Harold and co-workers (1957) could find no evidence for the transformation of cholesterol into cholestanol. The origin of cholestanol in these tissues is therefore still unknown. This also applies to the cholestanol of plumage where, in contrast to other tissues, it is more plentiful than cholesterol (Bolliger, 1960). At present it can only be stated that in feathers it is formed from an unknown precursor, as a by-product of the feather keratinisation. In the past, it has been shown that the soluble by-products of keratinisation may contain unusual types and amounts of metabolites (Bolliger and Gross, 1952). This also applies to the plumage of birds

which, in contrast to other tissues, contains, for example, large amounts of ammonia but no urea whatsoever (Bolliger and Gross, 1954). It may therefore be permissible, for the time being, to regard the constant presence of considerable amounts of cholestanol as a peculiarity of avian plumage. Cholesterol is the principal steroid in the blood of birds.

Nothing definite is known about the function of the cholestanol and its esters present in feathers; but one might reasonably assume that together with the other feather lipids of which they constitute about 10 p.c. they contribute to the waterproofing of the plumage. However, as shown in Table 2, aquatic and terrestrial birds possess about equal amounts of waterproofing agents, i.e. feather lipids, in their plumage. These lipids originate from the keratinisation process, which is basically similar in all birds examined and they have no connection with the preen or oil gland secretion, which is considered by many to be the source of the waterproofing material. This agrees with the observations of Fabricius (1959), Madsen (1941) and others who found that the removal of the preen gland from ducks just after hatching did not alter the waterproofing of their plumage. The feather lipids kept the ducks dry whether the preen gland was present or not. The secretion of the oil gland is therefore not required to make the plumage water repellent, but it may well constitute a supplementary waterproofing under unusual conditions. The main function of the preen gland's secretion seems to be to prevent a degeneration of the plumage which begins to occur about a month after removal of the oil gland (Madsen, 1941).

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STUDIES ON THE INNATE RESISTANCE OF MICE TO INFECTION WITH MOUSEPOX

I. RESISTANCE AND ANTIBODY PRODUCTION

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SUMMARY.

Strains of mice differ greatly in their susceptibility to mousepox. The highly resistant strain C57 Bl has been compared with a susceptible strain of mice with regard to virus growth and antibody production.

Differences in virus growth in the two strains of mice appeared at a certain stage of the infection, which was the time at which neutralizing antibody was demonstrable in the blood of resistant mice. Neutralizing antibodies, active immunity and hypersensitivity were shown to appear at least a day earlier in the resistant than in the susceptible mice.

The highest degree of resistance was found in young adult C57 Bl mice (2-3 months). Resistance was very low in new-born mice and very old mice (8-12 months), although death times were still delayed compared with those of susceptible mice of the same ages.

The resistance of C57 Bl mice to the lethal effects of infection with ectromelia virus appears to be due to a more effective immune response than that shown by mice of susceptible strains.

INTRODUCTION.

It has long been known that epidemics depend on the number of susceptibles in a population and that during the course of an epidemic of a lethal disease the more resistant individuals survive. It was early postulated, and convincingly demonstrated by Webster (1946) in experimental epidemics of mouse typhoid, that resistance to disease may be genetically determined. The varying resistance of rats to plague infection in Indian cities with high and low plague incidence (Sokhey and Chitre, 1937) suggested that genetic resistance to infection was also important under natural conditions. In recent studies on the myxomatosis epidemics in Australia, Marshall and Fenner (1958) demonstrated that in areas subjected to annual epizootics there was a steady increase in the genetic resistance of the rabbit population to this disease. Little is known, however, of the physiological basis of such genetic differences in susceptibility.

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In 1953, Trentin, in a report on mousepox outbreaks in the United States, mentioned that mice of the strain C57 Bl were more resistant than some other strains, and Briody (1959) has confirmed the high resistance of C57 Bl mice to this disease.

Mousepox, like myxomatosis, is a generalized virus disease, and its pathogenesis, elucidated by Fenner (1949a) closely resembles that of myxomatosis (Fenner and Woodroofe, 1953). Both of these viruses, moreover, are now classed as poxviruses (Fenner and Burnet, 1957). It was thought, therefore, that the resistance of C57 Bl mice to mousepox would be a convenient laboratory model for the study of the physiological basis of innate resistance to generalized viral infections.

Materials and Methods.

Diluents. Ca-Mg-Saline. 0·8 p.c. NaCl; 0·006 p.c. $CaCl_2$ $6H_2O$; 0·017 p.c. $MgCl_2$ $6H_2O$; 0·12 p.c. H_3BO_3; 0·005 p.c. $Na_2B_4O_7$ $10H_2O$ in distilled water (pH 7·2). *Gelatin Saline.* To the above Ca-Mg-Saline 0·5 p.c. gelatin was added as protein protectant ("Fine-leaf Gelatin E", Société des Prod. Chim., Coignet, Belgium).

Fowl Red Cells. Vaccinia-susceptible red cells were washed twice and made up to a 2·5 p.c. suspension in normal saline. This suspension could be used for five days without loss in sensitivity.

Sheep Red Cells. The red cells were washed twice and diluted to 3 p.c. in normal saline. Rabbit haemolysin was added and the cells incubated at 37° C. for 30 minutes before use.

Mice. The mice used were of the highly brother-sister inbred strain C57 Bl from the Animal Genetics Section, C.S.I.R.O., Sydney; and random bred multicoloured mice originating from the Walter and Eliza Hall Institute, Melbourne, which are referred to in this paper as stock mice. For certain experiments the inbred strains CBA and Bagg were also used.

All mice when used were about 2-3 months old, unless otherwise stated. Males and females were distributed similarly in each experiment. Although female mice were slightly less susceptible than males, this did not significantly influence the outcome of the experiments.

Virus. The Moscow strain of ectromelia (Fenner, 1949a) was used in most experiments. Highly infective virus stock suspensions were made from the spleens of mice moribund or dead after the massive intravenous or intraperitoneal injection of virus. The organs were ground in chilled mortars, and 1·0 ml. of gelatin saline added per spleen. The suspension was then treated for one minute with a Mullard 50W ultrasonic drill operating at maximal intensity, and centrifuged at about 1400 g. for five minutes on an angle centrifuge. Aliquots of the supernatant were stored at — 60° C.

In the experiments involving particle counts virus was purified by repeated genetron extraction (Gessler *et al.*, 1956).

The less virulent egg-adapted Hampstead strain of ectromelia (Fenner, 1949b) and cowpox virus were both used as chorioallantoic membrane suspensions.

Infectivity titrations were carried out either on the chorioallantoic membrane or in stock mice.

Pock titrations were carried out on the chorioallantoic membrane (CAM), the eggs being inoculated by the technique of Westwood, Phipps and Boulter (1957), and incubated for three days at 36° C. Titres are recorded as PFU (pock forming units) per ml. Ectromelia pocks are rather small compared with those of other poxviruses and counts are considerably more erratic. At least five eggs were used for each dilution and the counts were weighted by using only the three highest.

Serial tenfold dilutions of the material were injected into the footpads of groups of five or six stock mice. The animals were observed for 14 days and infection was recorded by either foot swelling or death, and when necessary liver and spleen were inspected for the typical ectromelia lesions. For different purposes four types of 50 p.c. endpoint were used in the infectivity titrations in mice. They were:

ID_{50} = 50 p.c. infectivity endpoint. These were calculated as described by Reed and Muench (1938).

LD_{50} = 50 p.c. lethal endpoint; i.e. the dose at which 50 p.c. of the mice died, calculated in the same way as the ID_{50}.

IT_{50} = 50 p.c. incubation time; the day on which 50 p.c. of the inoculated mice showed swollen feet.

ST_{50} = 50 p.c. survival time; the day on which 50 p.c. of the inoculated mice had died.

Haemagglutinin titrations were carried out in perspex trays. The material to be titrated was diluted out in doubling steps in 0·25 ml. of normal saline containing 1 p.c. rabbit serum to inhibit nonspecific lipid haemagglutination. 0·05 ml. of 2·5 p.c. vaccinia-susceptible fowl cells was added to each cup and after 20 minutes incubation at 37° C. the trays were shaken, and reincubated for 40 minutes. Partial agglutination was taken as the endpoint.

Complement fixing antigen was titrated in perspex trays by diluting it out in doubling steps in 0·25 ml. of CaMg saline. Ectromelia antiserum in excess and two haemolytic units of complement were added to each cup, and the whole was incubated overnight at 4° C. 0·025 ml. of 3 p.c. sensitized sheep cells was then added and the test incubated at 37° C. for thirty minutes, with a shake after the first 15 minutes. When the cells had settled at room temperature, the test was read taking 50 p.c. haemolysis as endpoint.

Antibody titrations.

Since the pocks produced by ectromelia virus are rather difficult to count on the CAM, and the yield of complement fixing (CF) and haemagglutinating (HA) antigen from infected membranes was rather low, cowpox virus, which is very closely related to ectromelia (Downie and MacDonald, 1950) was used for most antibody titrations. The white variant of cowpox (Downie and Haddock, 1952) produces large white pocks on the chorioallantoic membrane, which are much more readily identified and counted than those of ectromelia, and this strain was therefore used in pock neutralization tests. The natural cowpox virus produces haemorrhagic pocks on the membrane, and chorioallantoic membrane preparations give high haemagglutination and complement fixation titres. It was used for the haemagglutination inhibition and complement fixation titrations.

Serial tail bleeds from mice were obtained as described by Sobey and Adams (1955). All sera were inactivated at 56° C. for 30 minutes.

Haemagglutination inhibition titrations were carried out in perspex trays. Sera were absorbed with vaccinia susceptible fowl cells to remove any nonspecific haemagglutinins. Doubling dilutions of the test sera in 0·25 ml. volumes of 1 p.c. normal rabbit serum in normal saline were made and two haemagglutinating units of cowpox virus added to each cup. After allowing the antigen-antibody mixture to react for 20-30 minutes at 37° C. red cells were added and haemagglutination endpoints recorded as in the haemagglutinin titration.

Complement fixation tests were carried out like the complement fixing antigen titrations. Sera were diluted out in doubling steps in 0·25 ml. volumes of CaMg saline. Cowpox antigen in excess (i.e. more than three complement fixing units) and two haemolytic units of complement were added to each dilution, the sera incubated overnight at 4° C. and the indicator system added as described.

Neutralization tests. The sera were diluted as little as possible — in no case more than 1:8 — because the neutralizing power disappears rapidly with increasing dilution. White cowpox virus was added to each serum sample to give a final concentration of 50-100 pock

forming units per 0·1 ml. After 1½ hours incubation at 37° C. each virus-serum mixture was inoculated onto 6-10 eggs. Pock counts were "weighted" as described above and the neutralizing power was expressed as the percentage of pock forming particles inactivated, taking the pock counts produced by normal mouse serum-virus mixtures as the control.

Tissue cultures.

Monolayers of mouse embryo fibroblasts were prepared from 18-19-day-old embryos by methods similar to those commonly used for the preparation of monolayers of chick embryo fibroblasts (Dulbecco, 1952). The medium used consisted of 0·5 p.c. lactalbumin hydrolysate and 8 p.c. calf serum in Hank's balanced salt solution. For infectivity titrations 0·1 ml. inocula were added to the washed monolayers and allowed to adsorb for four hours, and then overlay prepared according to Franklin, Rubin and Davis (1957) was added. Plates were incubated in a humidified incubator gassed with 5° CO_2 and air, and plaque counts were made on the fifth day.

EXPERIMENTAL.

Comparison of the sensitivity of infectivity titrations.

Genetron-purified virus preparations were titrated in mice (footpad inoculation), on the CAM, on mouse embryo fibroblast monolayers, and on chick embryo fibroblast monolayers. The same preparations were examined under the electron microscope by Miss M. Briggs and particle counts made. The mouse infectivity titration method was the most sensitive one (Table 1) and almost every virus particle was infectious for mice. About one in ten particles produced a pock on the CAM and one in twenty a plaque on mouse or chick embryo cell monolayers.

TABLE 1.

Number of virus particles, determined by electron-microscopic counting, equivalent to various biological titration units.

Experiment	Mouse pad inoculation		Chorio-allantoic membrane (pfu)	Chick embryo fibroblasts (pfu)	Mouse embryo fibroblasts (pfu)
	ID_{50}	LD_{50}			
1	2·5	25	7	20	30
2	1	10	20	16	10
3	1	40	10	24	18
Mean	1·5	25	12	20	19

Infectivity and lethality of ectromelia for different strains of mice.

Thirty-two mice of comparable age of the strains C57 Bl, CBA, Bagg, and twenty-four stock mice were inoculated into the footpad with approximately 40 ID_{50} of the Moscow strain of ectromelia virus.

This dose killed all stock mice, 82 p.c. of the CBA, and 47 p.c. of the Bagg mice. All 32 C57 Bl mice survived. The 50 p.c. incubation time (IT_{50}) was

TABLE 2.

Mortality, 50 p.c. incubation time (IT_{50}) and 50 p.c. survival time (ST_{50}) of mice of different strains after the injection of 40 ID_{50} of ectromelia virus (Moscow) into the footpad.

	Stock	C57 Bl	CBA	Bagg
Number inoculated	24	32	32	32
Percentage killed	100	0	82	47
IT_{50} (days)	—	9½	9½	9½
ST_{50} (days)	9½	—	9½	>14

a little delayed in mice of the C57 Bl strain as compared with mice of the strains CBA and Bagg. Many stock mice died before showing any swelling of the foot. The survival time (ST_{50}) increased with increasing resistance (Table 2).

A titration was carried out in C57 Bl and stock mice by footpad inoculation, using tenfold dilutions of virus and twelve mice per group. The results are shown in Fig. 1. The infectivity endpoint (ID_{50}) was the same with both strains,

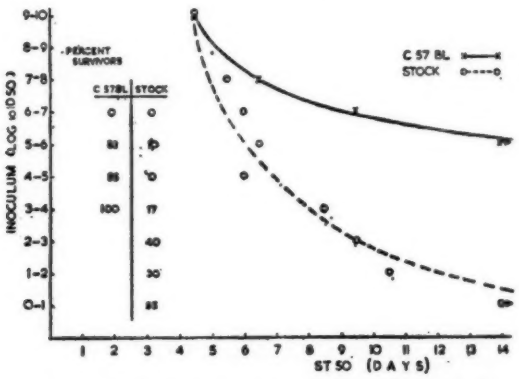

Fig. 1. Mortality and survival times (ST_{50}) after the inoculation of different doses of ectromelia virus into the footpads of resistant and susceptible mice.

whereas the 50 p.c. lethal dose (LD_{50}) for C57 Bl mice was a million times greater than the ID_{50}. In a large number of titrations of ectromelia in stock mice the LD_{50} was rarely greater than 10 ID_{50}, and in many cases was the same as the ID_{50}. The 50 p.c. survival times (ST_{50}) for the different doses of ectromelia have been plotted in Fig. 1, from which it is apparent that not only is the mortality lower in the resistant strain but also their ST_{50} is significantly higher.

Resistance to infection with ectromelia is therefore strain dependent, C57 Bl and stock mice showing extremes of resistance and susceptibility. Resistant mice are as readily infected as susceptible ones, but fewer C57 Bl mice die, and when death occurs it is delayed.

The course of the infection after injection of virus into the footpad.

Fenner (1949b) compared the course of infection after footpad inoculation of stock mice with two strains of ectromelia virus, the virulent Moscow strain and the attenuated Hampstead egg strain. He found that the time sequence of infection of different organs was much the same with these two strains of virus, but that the more virulent strain multiplied much more rapidly, and to higher titre, in the liver and spleen. The more severe skin rash and the higher mortality of the Moscow strain were ascribed to this.

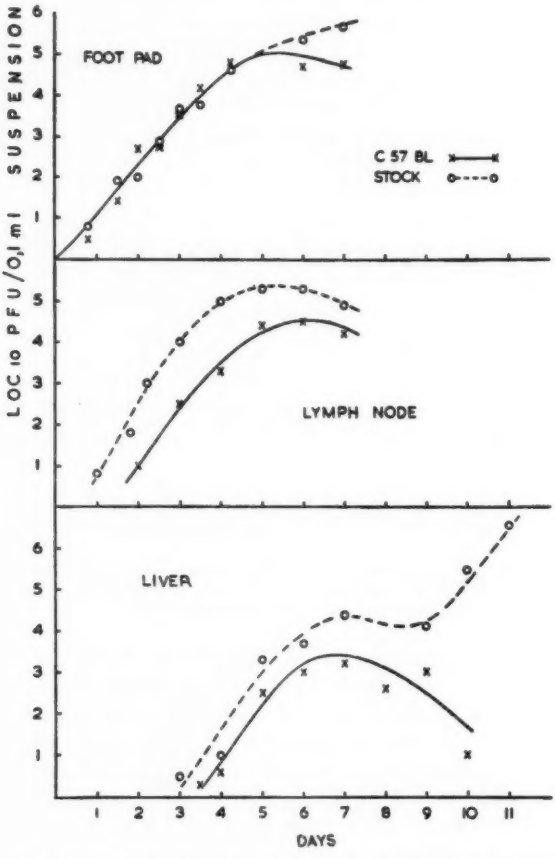

Fig. 2. Growth of ectromelia virus in the injected footpad, regional lymphnode, and liver of C57 Bl and stock mice after the injection of 300 ID_{50} into the footpad.

Since our interest was in the differences between different strains of host animal similar experiments were conducted using stock and C57 Bl mice and the Moscow strain of virus. About 300 ID_{50} were injected into the footpad of mice of both strains, and at various intervals 2-4 mice were sacrificed, and footpads, popliteal lymphnodes, livers and spleens removed separately. The tissues were pooled and titrated on the chorioallantoic membrane. The findings described below are based upon the accumulated data of these experiments (Fig. 2). Virus multiplied at the same rate in the footpads of both strains until the fifth day after infection. From then on there was no increase in titre in the resistant C57 Bl, while in the stock mice the titre continued to rise, although more slowly, until the end of the experiment. In the local lymphnode, on the other hand, virus was detected a day earlier in the susceptible stock mice than in the resistant mice, and throughout the experiment titres in the lymphnodes of the C57 Bl mice were only one-tenth as high as in the susceptible animals. Virus growth in the liver was similar in each strain of mice for the first five days, although the virus appeared a little later and titres were slightly lower in the resistant animals. After the sixth day, however, the titre in the resistant mice levelled out and began to fall, while in the stock mice there was a significant though only shortlived interruption in the growth rate of virus. The growth in the spleen followed very closely that in the liver. It was noted, however, that spleen titres are somewhat lower than the liver titre in the resistant strain and somewhat higher than liver titres in the susceptible ones (Table 3).

In another experiment a larger dose ($10^{7.3}$ ID_{50}) was injected into the footpad of stock and C57 Bl mice, and liver and blood growth curves were constructed. With the larger dose the differences in blood and liver titres of resistant and susceptible mice became apparent by the third day (Fig. 3).

In summary, virus growth was the same in the inoculated footpad of both strains of mice for the first five days. Virus appeared earlier in the draining lymphnode of the susceptible mice and stayed at a higher level throughout the experiment. Virus also appeared earlier in the livers of the susceptible mice

TABLE 3.

Virus content of liver and spleen of mice of different strains sacrificed at various times after the inoculation into the footpad of 10^3 ID_{50} of ectromelia virus (Moscow).

Day after injection	C57 Bl		Stock	
	Liver	Spleen	Liver	Spleen
4	2·3*	<2	1·7	3·5
6	4·2	3·8	6·9	7
8	3·2	2·7	7·9	8

* Expressed as $\log_{10}$ ID_{50} per organ.

but the growth rate was similar in both strains of mice until the sixth day, when the titres in the resistant mice levelled out and then fell rapidly, whereas the virus concentration continued to rise in the livers of the susceptible mice. With larger doses liver titres were identical during the early phase, but the different behaviour in the two strains was apparent on the third day. With the smaller doses the strain differences appeared earlier in lymphnode and spleen than in the footpad and the liver.

Influence of age upon susceptibility.

The virulence of mousepox in stock mice has been shown to change with increasing age (Fenner, 1949c). Very young and old mice had a higher mortality and shortened survival time as compared with mice eight weeks old.

The differences in the susceptibility of different strains of mice to ectromelia as they have been described above concerned young adult mice. In this age group the ID_{50} of Moscow ectromelia virus, after footpad inoculation, was the same for C57 Bl and stock mice, but the LD_{50} for C57 Bl was about a million times higher than for stock mice.

In a number of experiments 1-2-day-old stock mice and mice of the C57 Bl strain were injected subcutaneously with about 10^3 ID_{50} of virus. Deaths were recorded at 3-4 hourly intervals. Since there were some nonspecific deaths only mice dying after four days were taken to have died from ectromelia. In Fig. 4 (derived from three similar experiments) the survival times of 48 sucklings of each strain are shown. All mice of both strains died of ectromelia, but there was a significant difference in survival time. The ST_{50} of the C57 Bl mice was more than 12 hours longer than that of the stock mice and deaths among the C57 Bl were spread over a longer period.

In ageing mice the susceptibility to ectromelia increases. Thus 8-12-months-old mice of each strain and eight-weeks-old controls were inoculated with approximately 10^3 ID_{50} into the footpads. This dose, although not sufficient to kill C57 Bl mice two months old, killed 60 p.c. of the 8-12-months-old mice of this strain. All the stock mice died and the

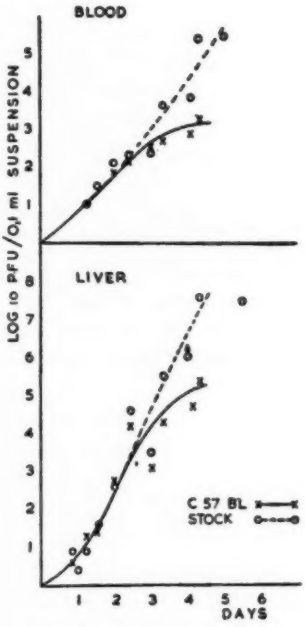

Fig. 3. Liver and blood titres after the injection of $10^{7.3}$ ID_{50} of ectromelia virus into the footpad of C57 Bl and stock mice.

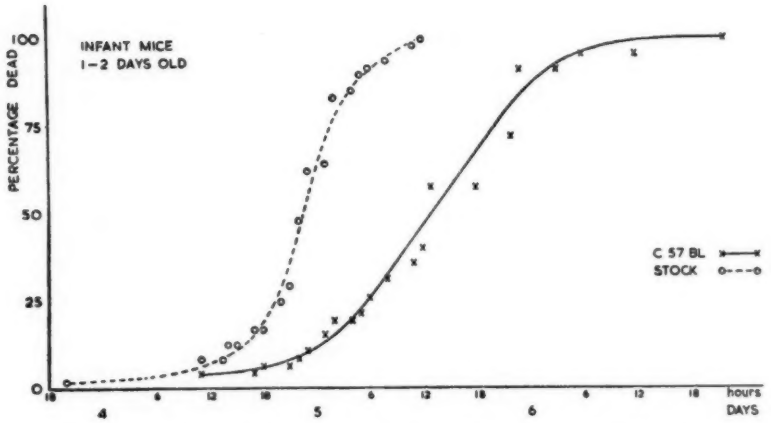

Fig. 4. Survival times of C57 Bl and stock baby mice after the subcutaneous injection of 10^3 ID_{50} of ectromelia virus.

older mice had a considerably shorter survival time (Table 4) than the eight-weeks-old controls. It was noted that the foot swelling following the inoculation started significantly *later* in the old mice regardless of strain as compared with the younger controls.

Thus, the difference in susceptibility to ectromelia is most obvious with mice two months old. Very young mice are very susceptible to ectromelia, and all suckling mice of both strains died, although the survival time of the C57 Bl was significantly longer than that of the stock mice. Old mice, also, are more susceptible than young adults, but again the C57 Bl were more resistant than stock mice.

TABLE 4.

Mortality and survival time of C57 Bl and stock mice of different ages after a small footpad injection of ectromelia virus (Moscow).

Age at inoculation	C57 Bl		Stock	
	Mortality	ST_{50}	Mortality	ST_{50}
1-2 days	48/48*	5½°	48/48*	5°
4-8 weeks	0/16	—	15/16	9
8-12 months	12/22	14	8/8	7

* $\frac{\text{No. mice dead}}{\text{No. inoculated.}}$

° days.

Elimination of differences in peripheral susceptibility by injection of the virus directly into the bloodstream.

The experiments described above show that the principal differences in the growth of virus in the two strains of mice are not seen until five days after the peripheral injection of a small dose of virus, suggesting that an immune response is involved. With a large dose, however, differences in the growth curves were apparent as early as three days after the injection. A very prompt immune response in resistant mice could still account for this early difference. Differences in the popliteal lymphnode titres, however, were already established 24 hours after the injection of a small dose of virus and although these differences could be due to a very early antibody response in the draining lymphnode, it is possible that it reflects a difference in cell susceptibility. Again, the experiments with baby mice suggested that the immune response does not by itself account for the difference in susceptibility.

It has been shown that after the peripheral injection of ectromelia virus, the virus multiplies first at the site of infection, then in the regional lymphnode from which it spills over into the bloodstream and enters the viscera (Fenner, 1949a).

Another experiment was therefore done, in which possible differences in peripheral tissue susceptibility were eliminated and the immune response minimized by injecting massive doses of virus intravenously. $10^{8.5}$ ID_{50} of ectromelia, strain Moscow, were given into the tail veins of stock and C57 Bl mice. The disappearance of the injected virus from the blood was followed by titrating serial tail bleeds on the chorioallantoic membrane. A second group of mice was sacrificed at various times after the injection and virus multiplication in the livers of mice of both strains compared. The remaining group of mice was kept under observation to compare their death times. There was no significant difference in the clearance of the injected virus from the blood (Fig. 5) nor in the growth of virus in the liver (Table 5). Mice were checked 4-6 hourly, but no significant strain difference in survival times was found (see upper part of curve in Fig. 6).

When, however, smaller amounts of virus were injected intravenously, a

TABLE 5.

Virus titres in livers of pools of three mice killed at various intervals after the intravenous injection of $10^{8.5}$ ID_{50} *of ectromelia virus (Moscow).*

Hours after infection	C57 Bl	Stock
10	5·9*	6·1
21	7·6	6·8
31	8·9	9·2
48	10·1	9·9

* Expressed as $\log_{10}$ ID_{50} per liver.

difference in the response of the two strains of mice became apparent. When doses of less than about 10^6 ID_{50} were given, so that mice survived longer than four days, there was a striking decrease in the mortality of C57 Bl mice and their survival time was significantly prolonged as compared with stock mice (Fig. 6).

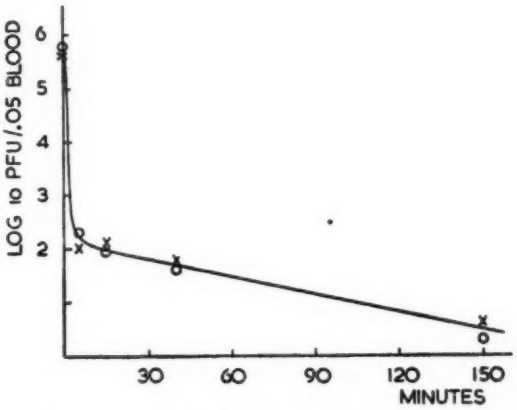

Fig. 5. Clearance of ectromelia virus from the blood of resistant and susceptible mice after the intravenous injection of $10^{8.5}$ ID_{50}. x: C57 Bl, o: stock mice.

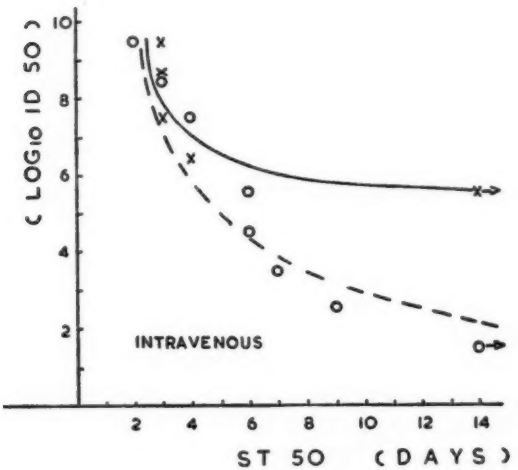

Fig. 6. 50 p.c. survival times after the intravenous injection of different doses of ectromelia virus into C57 Bl and stock mice. x —— x: C57 Bl mice. o - - - o: stock mice.

Thus, the difference in susceptibility to ectromelia of C57 Bl and stock mice was not seen when the virus was given directly into the bloodstream in doses large enough to kill the animals before the fourth day, but when smaller doses were given intravenously, so that mice lived more than four days, the greater resistance of C57 Bl mice became apparent.

Susceptibility of cultured cells from resistant and susceptible mice.

The above experiments provide the principal evidence against the hypothesis that differences in cell or tissue susceptibility are responsible for differences in host resistance to mousepox. Differences between the two strains of mice are not detectable until several days after infection, the exact time depending on the dose given. The possible role of cell susceptibility was further examined by testing virus growth in cells isolated from the body.

Monolayers of mouse embryo fibroblast cells were prepared from C57 Bl and stock mouse embryos, and were infected with ectromelia virus. The virus grew equally well in cells from mice of the two strains. The numbers of plaques formed by a given inoculum and the amount of virus produced per plaque were the same for the two types of cell. Thus, no difference was detectable in virus multiplication in embryo cells derived from mice of the two strains.

Attempts to infect cell suspensions and monolayers derived from adult mouse tissue were unsuccessful.

The production of antibodies.

The Moscow strain of ectromelia is so virulent that one infective unit often kills mice of the susceptible strain. For this reason the much less virulent egg adapted Hampstead strain was used in experiments in which the antibody response of C57 Bl and stock mice was compared.

Several experiments were carried out as follows. After a preliminary blood sample had been taken from the tail, about 10^4 ID_{50} of Hampstead virus were injected into the footpads of 2-4 mice of each strain. At suitable intervals the mice were bled

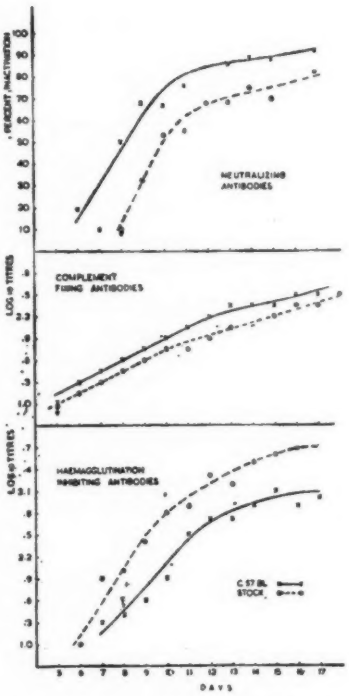

Fig. 7. Serum antibody titres after the injection of 10^4 ID_{50} of ectromelia virus (Hampstead egg strain) into the footpads of C57 Bl and stock mice.

from the tail and the bleeds of each group pooled. Sera were titrated for the different types of antibody, and the accumulated data are recorded in Fig. 7.

Haemagglutination inhibiting antibodies were found to be produced in higher titres in the susceptible than in the resistant mice. Although this difference was small during the earlier phase of antibody production, it became larger as antibody formation reached its peak.

The production of complement fixing antibodies did not differ very much in the two different strains, although it was slightly higher in the resistant mice. Neutralizing antibodies, however, were produced faster and reached higher levels in the resistant mice. Significant antibody titres (i.e. more than 50 p.c. inactivation) had been reached more than a day earlier in this strain.

Thus, resistant mice (C57 Bl) were better producers of neutralizing and possibly complement fixing antibodies than are susceptible stock mice. On the other hand, stock mice produced higher levels of HAI antibodies.

The onset of immunity following primary infection indicated by the response of the footpad to a large challenge dose of virus.

The production in mice of hypersensitivity of the delayed type following ectromelia infection has been described by Fenner (1948). This form of host response was compared in the two strains of mice. Groups of eight mice of each strain were sensitized (infected) with about 10^4 ID_{50} of ectromelia (Hampstead egg preparation) by injection into a front footpad. At various time intervals they were challenged by injection of about 10^9 ID_{50} of the Moscow strain into a hind footpad. The degree of footpad swelling was estimated by eye and scored accordingly. The results are recorded in Fig. 8.

There was no significant difference in the onset or rate of foot swelling in control mice of the two strains which received only the challenge injection and these mice died before maximal swelling developed.

In susceptible mice challenged four days (3 days 19 hr.) after the initial sensitizing injection the rate of foot swelling was the same as in the controls. They survived, however, and went through the stages of maximal swelling, necrosis and loss of the foot. Mice challenged at five days showed a more gradual swelling and only an occasional mouse went through the maximal stages of swelling and amputation. The longer the time between sensitization and challenge the less extensive was the foot swelling and the earlier its onset. In mice challenged at 10½ days a hypersensitive response was observed in the form of an early, transient, swelling of the injected foot.

In resistant mice challenged four days after sensitization there was already much less extensive foot swelling than in susceptible and control mice, and only about half the mice lost their foot. This difference in the severity of foot swelling of resistant and susceptible mice was greater still when challenge was on the

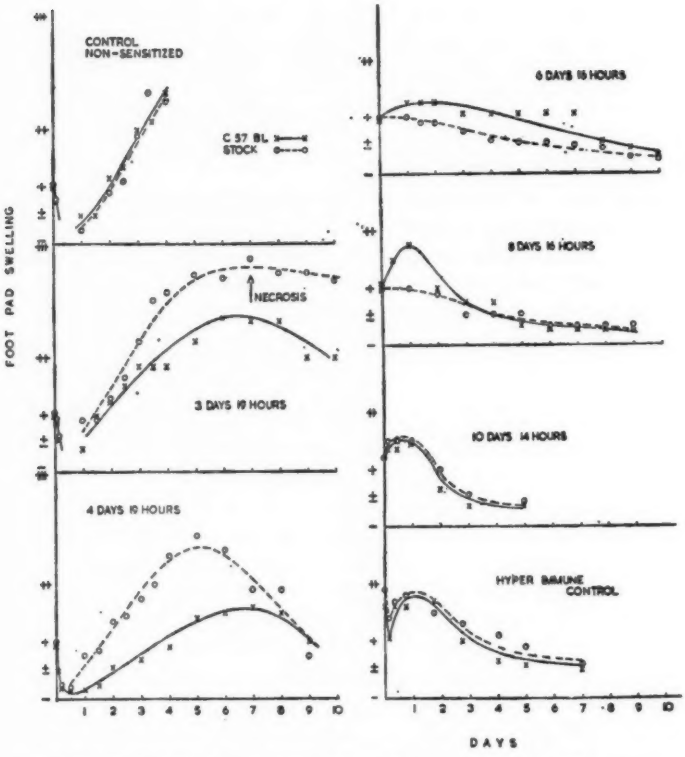

Fig. 8. Demonstration of immunity and hypersensitivity by the response of the foot to the challenging injection of $> 10^9$ ID_{50} of ectromelia virus (Moscow strain) in mice infected at various times previously with 10^4 ID_{50} of ectromelia (Hampstead egg strain).

fifth day but then declined. By the ninth day the hypersensitive response had appeared.

The hypersensitive response was also obtained when mice hyperimmune to ectromelia were challenged in the same way, and there was no significant difference between hyperimmune suceptible and resistant mice.

It can be seen, then, that four days after a small injection of virus, antibody production has already developed sufficiently for mice of either strain to withstand the lethal effect of a large challenge dose of virus. If the diminished foot swelling produced by the challenge on the fourth day is taken as an index of antibody production, this antibody response appears a day earlier in resistant mice. Hypersensitivity also appears approximately a day earlier in the resistant

animals. The difference between the strains is apparent only as a difference in the time of onset of the immune response. There is no difference detectable, by the criteria employed, in the final extent of the immune or hypersensitive response.

This experiment shows by direct challenge infection that immunity to ectromelia virus is produced significantly earlier in mice of the resistant C57 Bl strain.

Further observations on immune response as indicated by spleen size.

Observations on the spleens of ectromelia infected mice suggested that there was a relationship between spleen size and the stage of the disease. The spleen as a major antibody producing organ is known to increase in size in direct relation to antigenic stimulation and antibody production (Wiseman, 1931; Taliaferro, 1956). Therefore the spleen size of resistant and susceptible mice was compared during the course of a standard infection.

For this experiment CBA mice were chosen as the susceptible strain, since normal animals of this strain had much the same sized spleens as C57 Bl mice, whereas stock mice had much larger spleens. Groups of mice were injected into the footpad with about 400 ID_{50} of the Moscow strain of ectromelia. At various times after inoculation groups of 3-4 mice were killed, always in exactly the same way, with ether. The two main diameters of the spleen (a + b) were recorded. The approximate spleen volume was calculated according to the formula $(a \times b)^{\frac{3}{2}}$ and plotted in Fig. 9.

In the susceptible mice (CBA) the spleens increased in size during the first four days, then in most cases decreased quite strikingly. Deaths occurred from the eighth day onward and all dead mice had small spleens. A small proportion of the mice killed after the fourth day showed an enlarged spleen. All mice surviving longer than ten days also had greatly enlarged spleens.

Some of the CBA mice were expected to survive the dose of virus given and it is possible that CBA mice, sacrificed between the sixth and the tenth day, which had large spleens would have survived.

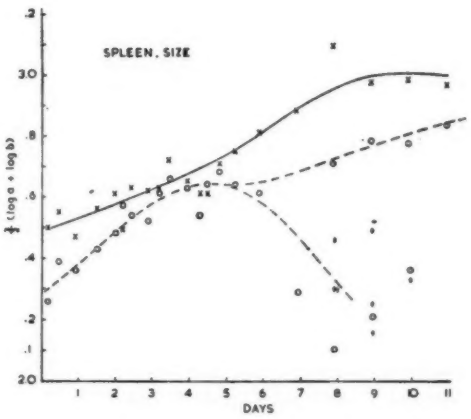

Fig. 9. Changes in spleen size of CBA and C57 Bl mice during the course of a standard infection after the injection of 400 ID_{50} of ectromelia virus into the footpad. x —— x: C57 Bl. o - - - o: CBA mice. †: CBA mice examined after death due to ectromelia.

In the resistant mice the spleen size increased slightly during the first four days. By the fifth day a very marked increase began and continued throughout the period of observation. All mice survived and all had greatly enlarged spleens during the later stages of the disease.

Thus a marked difference in spleen size of the two strains of mice became noticeable five days after injection, resistance being accompanied by continued increase in spleen size.

DISCUSSION.

There can be no doubt that heredity plays an important rôle in the resistance to mammalian viral infections (Gowen, 1948). Two principal factors have been shown to be of importance for the inherited differences in susceptibility: firstly, a difference in the suitability of the host tissue for virus growth, and, secondly, a different host response.

Webster and Johnson (1941) showed that St. Louis encephalitis virus multiplied at a different rate in brain cell cultures from mice selected for resistance or susceptibility to this virus. Further evidence for differences in cell susceptibility has been produced by Sabin (1954) who demonstrated that the growth of yellow fever virus and antigenically related viruses was depressed in a resistant mouse strain. Virus multiplied freely in tumour cells transplanted from susceptible to resistant mice, and the administration of cortisone did not affect virus growth, thus indicating that in this case antibody production was of only minor importance as the physiological basis of innate resistance.

Resistant mice were as sensitive to infection with ectromelia as susceptible ones. Differences in resistance due to different cell susceptibility should become apparent from the very beginning of virus growth, while a different immune response would develop only after some delay. It has been shown that virus growth in the footpad was the same in each strain during the first five days after the injection of a moderate dose of virus, and the same was true of virus growth in the liver. This suggests that an immune response is involved. Further, experiments with ectromelia virus in cultured cells obtained from C57 Bl and stock mice, showed no differences in cellular susceptibility. Thus in the present instance no evidence has been obtained that cellular susceptibility plays any part in the observed difference in the innate resistance of C57 Bl and stock mice to mousepox.

All the evidence provided by the present experiments points to the immune response as the operative factor in the different genetic resistance of C57 Bl and stock mice to ectromelia infection. Direct measurements of neutralizing antibody and of actively acquired immunity showed that C57 Bl mice responded earlier, and more effectively, than did stock mice. The differences in the growth curves of virus in footpad, liver, lymphnode and spleen of resistant and susceptible mice all point to the importance of the earlier appearance of antibody in C57 Bl mice.

The time at which differences in growth appeared varied with the organ and the dose. Both the local lymphnode and the spleen are important sites of antibody production, and in these organs the effect of antibody was apparent earlier than in the footpad and liver, in which antibody production does not occur. With large inocula the difference in the growth curves appeared earlier, due presumably to the earlier and more vigorous stimulation of antibody production.

With moderate doses of virus inoculated into the footpad several differences between resistant and susceptible mice appeared on the fifth or sixth day. Serum neutralizing antibodies were now detectable in C57 Bl mice, the growth curves of virus altered, and the differences in spleen size and active immunity were now established. All these changes can be ascribed to differences in the capacity for antibody production, which thus appears to be the physiological basis of the difference in innate resistance of C57 Bl and stock mice to ectromelia infection.

If it is a difference in antibody response which is responsible for the difference in susceptibility to ectromelia of the two strains of mice, this difference in response would be insignificant in very young mice, maximal in young adults in the "prime of life" (Burnet, 1952), and decrease in ageing mice. This has been shown to be the case. However, even in mice 1-2 days old, there was a delay in the death times of infant C57 Bl mice compared with infants of the stock strain. There are indications that baby mice can be immunized to eastern equine encephalomyelitis virus when only two days old (Morgan, 1941) and, if it is assumed that the rejection of homografts is an immunological response, then C57 Bl mice respond by the second day of life (Gross, 1950).

Throughout this discussion it has been assumed that neutralizing antibodies are important in determining the course of mousepox in infected mice. Some observations with human cases of agammaglobulinaemia, however, suggest that circulating antibodies may not be the only, or indeed the most important factor, in determining recovery from viral infections. Whether this be the case or not it seems certain that the physiological basis for the observed difference in resistance of C57 Bl and stock mice to mousepox is the more efficient immunological response of the former, using the term to include cellular as well as humoral factors.

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STUDIES ON THE INNATE RESISTANCE OF MICE TO INFECTION WITH MOUSEPOX

II. ROUTE OF INOCULATION AND RESISTANCE; AND SOME OBSERVATIONS ON THE INHERITANCE OF RESISTANCE

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(*Accepted for publication 2nd March, 1960.*)

SUMMARY.

The response of mice to ectromelia virus depends on the route of injection and the differences between C57 Bl and a susceptible strain of mice have been studied using different routes of injection.

The difference in susceptibility between C57 Bl and stock mice appears earliest following the intravenous injection of ectromelia virus, and following splenectomy this difference is abolished.

The strain difference is most pronounced when the virus is injected into the footpad. It is reduced to a minimum after intracerebral injection, probably because not enough antibody reaches the brain tissue. All resistance is abolished after intraperitoneal injection and for this no explanation is advanced.

Following the intranasal instillation of virus, C57 Bl mice are less resistant because they die of the pneumonia which follows a vigorous local response in the lungs. They succumb to doses of virus which are not lethal by the footpad route, and with larger doses they even die earlier than stock mice.

The difference in susceptibility following different routes of inoculation can be explained in terms of the difference in effectiveness of the antibody response and it has been shown that resistance is inherited as a single, autosomal, dominant factor.

INTRODUCTION.

In the preceding paper it was demonstrated that the greater resistance of C57 Bl mice to mousepox (compared with certain other strains of mice) was due to their more effective immune response. It was found, however, that there were apparent anomalies in the pattern of resistance when different routes of inoculation were used, and a more detailed study of the effect of the route of inoculation on resistance was therefore made.

The inheritance of resistance to mousepox was studied by breeding experiments.

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Materials and Methods.

The materials and methods used are set out in the preceding paper (Schell, 1960).

EXPERIMENTAL.

Groups of mice of C57 Bl (resistant) and stock (susceptible) mice were inoculated by various routes with different doses of ectromelia virus, strain Moscow. Deaths were recorded daily for a fortnight. The ID_{50} and LD_{50} end-points and the 50 p.c. survival time (ST_{50}), were calculated as described in the preceding paper.

The results are recorded in Tables 1 and 2 and in Figs. 1 and 2.

In the previous paper (Schell, 1960) footpad inoculation was used as the standard method of inoculation, since it is likely that natural mousepox is contracted through minute skin lesions (Fenner, 1947). The results obtained by

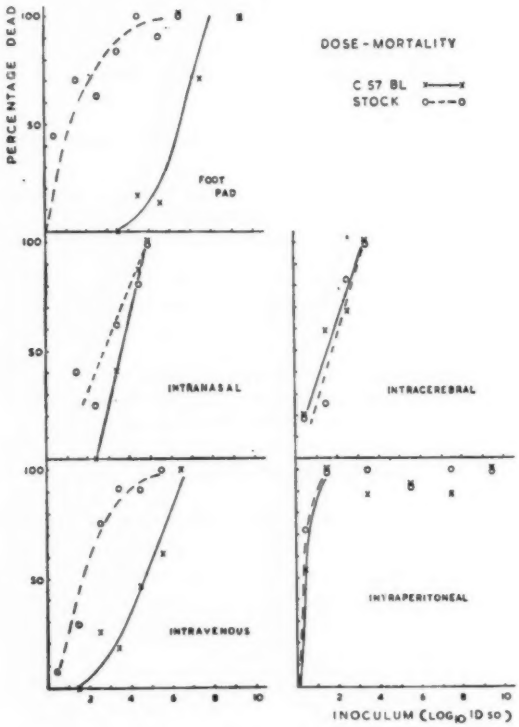

Fig. 1. Mortality of C57 Bl and stock mice following the injection of different doses of ectromelia virus (strain Moscow) by various routes.

TABLE 1.

Mortality and survival times of C57 Bl and stock mice following the injection of different doses of ectromelia virus (strain Moscow) by various routes.

Strain	Inoculum ($\log_{10}$ ID_{50})	Footpad		Intravenous		Intranasal		Intracerebral		Intraperitoneal	
		mortality	ST_{50}	mortality	ST_{50}	mortality	ST_{50}	mortality	ST_{50}	mortality	ST_{50}
C57 Bl	9-10	6/6+	5×	12/12	3	—	—	—	—	12/12	2
	8-9	5/7	6-7	9/9	3	6/6	4	—	—	—	—
	7-8	—	—	15/15	3	11/11	6	5/5	4	16/18	4
	6-7	8/8	7	5/5	4	8/8	6	10/10	5	—	—
	5-6	2/15	>14	10/16	>14	12/12	6	13/13	5	17/18	6
	4-5	3/18	>14	7/15	>14	13/15	9	20/20	7	—	—
	3-4	0/15	>14	2/11	>14	7/17	>14	11/11	5	16/18	8
	2-3	0/6	>14	3/12	>14	0/11	>14	11/16	9	—	—
	1-2	0/6	>14	0/5	>14	0/5	>14	3/5	9-10	4/4	8
	0-1	0/6	>14	0/5	>14	—	—	1/5	>14	6/11	8
Stock	9-10	6/6	5	12/12	2	—	—	—	—	22/22	2
	8-9	—	—	9/9	3	5/5	7	—	—	—	—
	7-8	3/3	6	10/10	4	11/11	6	5/5	4	18/18	4
	6-7	3/3	6	—	—	10/10	6	11/11	5	—	—
	5-6	9/10	7	5/5	6	12/12	7	11/11	5	17/18	6
	4-5	14/14	6	10/11	6	12/15	8-9	17/17	6	—	—
	3-4	14/17	9	11/12	7	10/16	10	12/12	8	18/18	7
	2-3	19/30	10	16/21	9	3/12	>14	14/17	8	—	—
	1-2	51/74	11	4/14	>14	2/5	>14	1/4	>14	4/4	8
	0-1	46/103	>14	1/15	>14	—	—	1/5	>14	8/11	8-9

+ no. mice dead/no. mice inoculated.
× days.
— no data available.

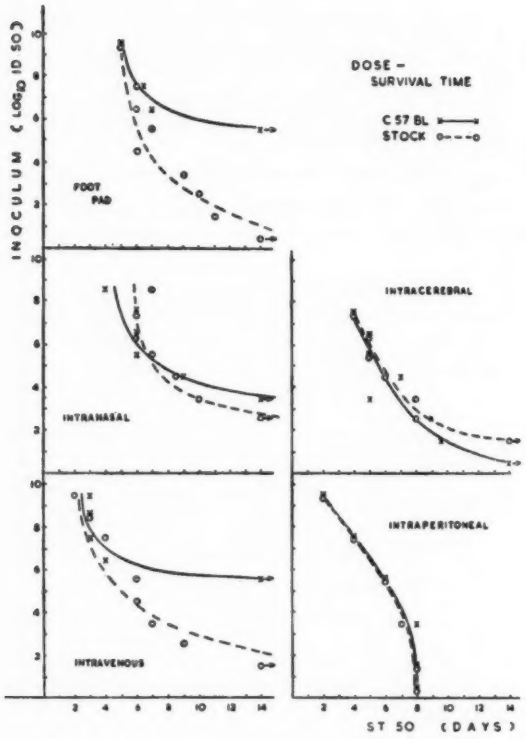

Fig. 2. Survival time (ST_{50}) of C57 Bl and stock mice following the injection of different doses of ectromelia virus (strain Moscow) by various routes.

other routes of inoculation will therefore be compared with the differences in resistance demonstrable by footpad inoculation, and explanations for discrepancies sought in the course of infection following different routes of inoculation.

Footpad inoculation.

As in previous experiments, there were striking differences in the response of C57 Bl and stock mice to footpad inoculation. Whereas C57 Bl mice are infected by the same minimal dose of virus, the lethal dose (LD_{50}) was very much greater for these mice than for stock mice (Table 2). The occurrence of a few survivors at most dose levels amongst the stock mice, in contrast to the sharp transition from non-lethal to lethal infections with increasing doses among the C57 Bl mice (Fig. 1), probably reflects the greater genetic heterogeneity of the former.

TABLE 2.

Effect of route of inoculation of ectromelia virus on LD_{50} and survival time in C57 Bl and stock mice.

Route of injection	LD_{50}		ST_{25} (Max. dose)	
	C57 Bl	Stock	C57 Bl	Stock
Footpad	5·4+	1·3+	5°	5°
Intravenous	4·0	2·0	2	2
Intranasal	3·7	3·1	4	6
Intracerebral	1·3	1·8	2	2
Intraperitoneal	0	0	2	2

+ $\log_{10}$ footpad ID_{50}/LD_{50}.
° days.

Differences in the survival time of fatal cases amongst the two strains of mice appeared when mice lived longer than six days, i.e. at dose levels less than 10^8 ID_{50}. Presumably after six days the immune response has developed sufficiently in C57 Bl mice to affect the course of the disease in these mice. It will be recalled that circulating antibody was first demonstrable in C57 Bl mice on the sixth day after footpad inoculation.

Intravenous inoculation.

When ectromelia virus is injected intravenously the stages of peripheral growth are eliminated and the virus is introduced directly to the major central sites of virus multiplication, the liver and spleen. The maximum dose therefore killed both strains of mice three days earlier when injected intravenously than when given by the footpad route (Table 2).

With doses larger than 10^7 ID_{50} no differences were found in the survival times of the two strains of mice (Fig. 2). Presumably with these large doses multiplication in the liver reaches a lethal level before the immune response can have an appreciable effect. With doses less than 10^6 ID_{50} mice survived more than four days and now differences between the strains were apparent. This indicates that an effective antibody response had developed in C57 Bl mice by the fourth day, as compared with six days by footpad inoculation. Presumably the introduction of virus directly into the spleen results in an earlier and more effective antibody response than occurs after footpad injection. The importance of the spleen in resistance to intravenously injected virus was shown in the following experiment. Splenectomy was carried out on groups of ten mice of each strain, and sham laparotomy on the controls. Next day all were inoculated intravenously with 10^4 ID_{50} of virus, with the results shown in Table 3. Clearly, splenectomy rendered C57 Bl as susceptible as normal stock mice to the intravenous injection of moderate doses of virus.

The efficiency of the immune response after intravenous injections of virus

TABLE 3.

Abolition of resistance to the intravenous injection of a moderate dose (10^4 ID_{50}) of ectromelia virus following splenectomy.

	C57 Bl		Stock	
	Splenectomy	Control	Splenectomy	Control
No. mice dead / No. inoculated	9/9	1/5	10/10	22/22
ST_{50} (days)	7	—	7½	7

is also indicated by the fact that virus injected in this way to stock mice is 10-20-fold less lethal than by footpad injection (Table 2) and produces a greater antibody response than in any other route of injection (Table 6).

Surprisingly, in C57 Bl mice the lethality was greater when virus was given intravenously than when it was injected into the footpad.

Intranasal inoculation.

When different doses of virus were instilled intranasally the difference in susceptibility between the two strains was not so marked as following intravenous or footpad injection (Table 2, Figs 1 and 2). Two points are worthy of comment. Firstly, with small doses the mortality amongst stock mice was significantly greater than among C57 Bl mice, this difference being apparent with doses such that mice died later than the eighth day. Secondly, and rather unexpectedly, it was found that C57 Bl mice died earlier than stock mice when larger doses had been inoculated. It is known (Kikuth and Gönnert, 1940) that massive intranasal instillation of ectromelia virus may produce a very severe local reaction. There may be complete consolidation and death as a result of extensive pulmonary exudation and cellular infiltration. The earlier deaths of C57 Bl mice with massive doses of virus could thus be due to a more rapid or a more vigorous local response of mice of this strain. With smaller intranasal doses this severe local reaction would not occur, and the better antibody response of resistant mice then would result in lower mortality and longer survival times than in stock mice.

Evidence that this was so was obtained in an experiment where mice of both strains were given various doses of virus intranasally and macroscopical liver and lung lesions were scored after death. The results are shown in Table 4.

In C57 Bl mice lung lesions were extreme in mice dying early and pronounced in those dying late. In none of these were there extensive liver lesions, and death after intranasal infection of C57 Bl mice appeared always to be due to pulmonary consolidation. In stock mice, on the other hand, death was attributable to pulmonary consolidation up to the fifth day only. Thereafter liver lesions were severe and probably constituted the main cause of death.

TABLE 4.

Post-mortem appearance of lung and liver of C57 Bl and stock mice following the intranasal instillation of ectromelia virus.

Days after inoculation	C57 Bl		Stock	
	liver	lung	liver	lung
3	−	+++	ND	ND
4	−	+++	±	+++
5	±	+++	++	+++
6	±	+++	ND	ND
7	+	++	++	+
8	(+)^r	++	+++	+
9	(+)^r	++	+++	−
10	(+)^r	++	+++	−

− macroscopically unchanged.
ND no data.
+++ maximum change.
(+)^r apparently recovered.

Intracerebral injection.

No strain differences were apparent in the response to intracerebral injection (Tables 1 and 2, Figs. 1 and 2), and the LD_{50} was between 10 and 100 footpad ID_{50}. It was thought that death was perhaps due to the growth of virus in the brain, and evidence that this was the case was obtained when livers and brains of mice of both strains were titrated at the time of death following the intracerebral inoculation of different doses of virus. None of the sixteen livers titrated had titres in the usual range (about 10^9 ID_{50}) found in mice dying after footpad inoculation, whereas all brain titres were much higher, whatever the dose of virus injected, than those seen following extraneural inoculation.

Even the least virulent of the extromelia viruses, strain Pittsburgh,[2] killed when it was injected in moderate doses into the brain (see below).

Intraperitoneal injection.

With both strains of mice very high mortalities were recorded after intraperitoneal injection, and the LD_{50} dose was virtually the same as the ID_{50} dose determined by footpad inoculation. With both strains it appears that one viable particle of Moscow ectromelia virus inoculated intraperitoneally is lethal. The dose-survival time curve (Fig. 2) shows no upward concavity after intraperitoneal inoculation of the virus, in contrast to the regular deviations from linearity seen (especially with C57 Bl mice) after all other routes of inoculation.

In contrast to the observations by Higginbotham and Dougherty (1955) that most of the staphylococci injected intraperitoneally into rabbits reached

[2] Originally supplied by Dr. G. Ruckle.

the bloodstream within half an hour after injection and did not disappear again, less than 0·1 p.c. of the intraperitoneally injected virus was ever detectable in the bloodstream, and none was recovered after six hours (Table 5).

TABLE 5.

The appearance of virus in the blood following intraperitoneal injection of 10^7 pock forming units of ectromelia (Moscow).

Time after injection	Virus amount in the blood
5 minutes	3·0+
10 minutes	3·8
20 minutes	3·5
1 hour	3·4
4 hours	2·0
6 hours	<1·7

+ $\log_{10}$ PFU/1·5 ml. of blood.

The target organ for virus growth following intraperitoneal injection was thought to be the liver. Intraperitoneally injected mice never died later than intravenously injected ones, and it was thought possible that the virus reached its target organ directly *via* the peritoneum. Therefore, marginal sections of the livers of intraperitoneally injected mice were stained with fluorescein-coupled antibody. Antigen appeared considerably later in these livers than after intravenous injection, and it is therefore suggested that organs other than the liver may be involved by the intraperitoneal injection of virus and lead to death.

The antibody response after injection by various routes.

The conclusion was reached earlier that the major reason for the difference in the response of C57 Bl and stock mice to footpad inoculation of ectromelia virus was the earlier and more effective immune response in the former group. Moreover, differences in the immune response after injection of virus by different routes might explain the observed differences in lethality. Experiments were therefore conducted to determine whether there were differences in the antibody response of stock mice after various methods of inoculation. For this purpose the least virulent available strain of ectromelia virus, Pittsburgh, was used and doses of 10^4 ID_{50} were injected by all routes. Pooled sera were obtained from survivors by tail bleeds on the eighth, tenth, and twelfth day and pock neutralising antibody titres determined. All mice injected intracerebrally died by the seventh day, and intranasally injected mice became sick and several died during the experiment. The results are shown in Table 6.

Obviously, the antigenic stimulus was the most effective following intravenous injection. Antibody response was rapid and efficient. Following intraperitoneal and footpad injection the antibody response was somewhat weaker and less uniform, and the response to intranasal injection was very poor.

TABLE 6.

The production of pock neutralising antibodies in stock mice following the injection of ectromelia virus (strain Pittsburgh) by various routes.

Days	IV		IP		FP		IN	
	a	b	a	b	a	b	a	b
8	54+	43	8	34	0	40	0	13
10	94	87	27	63	0	58	ND	27
12	96	95	37	81	ND	70	ND	ND

a, b pools of sera of three mice.
+ p.c. inactivation.
ND no data, no survivors.

The Inheritance of Resistance.

To analyse the inheritance of resistance several cross and back cross experiments were carried out.

Using C57 Bl and stock mice, a male of each strain was mated with two females of the other strain. First generation offspring (F1) were back crossed to the parental strains. All mice were given a standard challenge injection of $10^{3.5}$ ID_{50} of the Moscow strain of ectromelia into the footpad when six to twelve weeks old, and compared with controls of the original resistant and susceptible strains.

No relationship between the resistance factor and sex was found and data were therefore pooled.

The F1 generation of the cross stock × C57 Bl was uniformly resistant, indicating that resistance was dominant and, naturally, the back cross to the resistant parent produced again resistant offspring. When, however, the F1

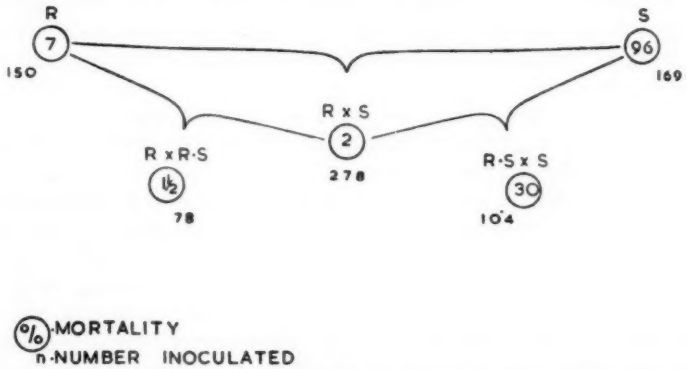

Fig. 3. The mode of transfer of the resistance factor in C57 Bl mice. R = resistant (C57 Bl); S = susceptible (stock).

generation was crossed back to the susceptible parent, susceptibility was not re-established to the 50 p.c. level as would have been expected if stock mice were homozygous for a susceptibility factor, and only 30 p.c. of the challenged mice died (Fig. 3). This intermediate behaviour is not unexpected in view of the genetic heterogeneity of stock mice, other evidence for which was presented earlier.

The data show that resistance is inherited as a single, dominant, autosomal factor and that mice of the strain C57 Bl are homozygous in this regard.

DISCUSSION.

Most of the apparently anomalous results of challenge infection by different routes of inoculation can be explained on the basis that the major attribute contributing to the recovery of mice from mousepox is the immune response, which was demonstrated to be earlier, and more vigorous, in C57 Bl than in stock mice.

Survival or death depends primarily upon the inter-relationship between the rate of multiplication of virus in the target organ, and the speed and intensity of the immune response. Viewed in this context, pad inoculation (or natural infection) gives the maximum opportunity for the very effective immune response of C57 Bl mice to alter the course of the infection, for while an early antibody response originates in the local lymphnode growth in the target organ (the liver) does not occur until a later stage of the disease. When virus is injected intravenously the antibody producing mechanism is stimulated from the start. Growth in the liver, however, also begins straight away, and unless antibody production is rapid enough to halt this growth, death will occur. If it is assumed that there is a maximum rate of antibody production, it is clear that as the dose increases, growth in the liver will be more likely to outstrip antibody production. Thus the larger the intravenous dose the less effective the antibody response, and the more susceptible the mouse will be to intravenous compared with footpad injection. This is in fact what is observed for C57 Bl mice as the intravenous dose is increased above about 10^3 ID_{50}, and stock mice are then as susceptible to intravenous as to footpad injection. With smaller intravenous doses the earlier and more vigorous immune response which follows this route of injection (Table 6) is effective, for all C57 Bl mice now survive, and fewer stock mice die than by footpad injection.

The failure to find any effect of strain upon the response to intraperitoneal inoculation, and the extreme lethality of this route of inoculation, are somewhat harder to interpret, although similar observations of a decreased resistance to bacteria after intraperitoneal injection have been made (Dutton, 1955). The possibility of a more rapid invasion of the liver has been excluded by the results of fluorescent antibody staining. Perhaps virus injected into the peritoneal cavity reaches some other essential organ which it damages irreversibly before antibody production has sufficiently developed.

Intranasal and intracerebral injections resemble each other in that each route of inoculation introduces virus into a target organ other than the liver. While intracerebral injection leads to a large overflow of virus into the vascular system (Cairns, 1950) multiplication of the virus in the ependymal and meningeal cells (Mims, 1960) proceeds virtually uninfluenced by circulating antibody (Morgan *et al.*, 1942) and death is due to the cerebral lesion.

The immune response of mice after intranasal injection appears to contribute to the death of C57 Bl mice by what can be regarded as a hypersensitive response in a vital organ. This explains the earlier deaths of C57 Bl mice after large doses, and their deaths from pulmonary consolidation after moderate doses. These deaths after moderate doses are perhaps made more likely by the poor antibody response occurring after intranasal injection (Table 6) and the small amounts of this antibody which reach the respiratory epithelium (Fazekas de St. Groth and Donnelley, 1950). In stock mice, on the other hand, only large doses kill by the local reaction to virus multiplication in the lung, and smaller doses by invasion of the liver and the usual later death from liver damage.

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EVAPORATIVE WATER LOSS AND TEMPERATURE REGULATION IN THE MARSUPIAL *SETONYX BRACHYURUS*

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SUMMARY.

Salivation on the fur does not affect the temperature regulation, at high environmental temperatures, of the quokka *Setonyx brachyurus*. Evaporative water loss is reduced in *Setonyx* after dehydration, temperature regulation being correspondingly poorer. This cannot be related to the reduction in food intake observed in dehydrated animals.

INTRODUCTION.

Temperature regulation in marsupials is of interest with respect to both phylogeny and ecology. Phylogenetically, marsupials are intermediate between the monotremes which are heterothermic and placentals which are homoiotherms (see Davson, 1959). Many marsupials live in areas where temperature regulation may be a problem due to high environmental temperatures and a limited water supply.

Early reports indicated that marsupials were not as efficient homoiotherms as placentals (Martin, 1902). This has since been found not to be generally true. Bentley (1955), for instance, has shown that the Rottnest quokka *Setonyx brachyurus* is a very efficient homoiotherm. Bartholomew (1956) and Robinson and Morrison (1957) have confirmed this, but claim that to maintain a constant body temperature at high environmental temperatures *Setonyx* and other marsupials depend to a considerable extent on evaporation of saliva from their fur. Their suggestion is examined in more detail in this paper, with respect to *Setonyx*.

Many Australian marsupials living in hot arid regions with limited water supplies would presumably be at some disadvantage in evaporative cooling of their body. Man, apparently, has no physiological control over this form of water loss (Adolph, 1947), but evaporative loss is reduced in the camel and the rat when dehydrated (Schmidt-Nielsen, Schmidt-Nielsen, Houpt and Jarnum, 1956; Dicker and Nunn, 1957). In the camel, temperature regulation is poorer under such conditions (Schmidt-Nielsen and Schmidt-Nielsen, Houpt and Jarnum, 1955), and the same could be expected in the rat. The possibility of an adaptive reduction in evaporative loss has been studied in *Setonyx*.

Methods.

General. The animals were originally members of the wild population on Rottnest Island and were allowed ample time to adjust to a life in captivity before use. They weighed from 2·8 to 4 kg. and there were equal numbers of males and females in each experiment.

Except where specifically stated the humidity control was set at 55 p.c., but this was not very efficient. On different days at any particular temperature the maximum differences in humidity were of the order of 10 p.c.

In order to allow for this day to day variation in humidity and other factors that may vary similarly all the experiments were performed using a crossover design.

The animals were allowed a week to recover from the starvation and dehydration procedures before continuing with the next part of the experiment. In the experiment where fur licking was prevented, three days separated the different trials.

Evaporative water loss. The animals were placed in metabolism cages and weighed on a balance accurate to 0·5 gm. before and after the experiment. The weight of the urine and faeces collected during the experimental period was subtracted from the total weight loss of the animal, giving the loss by evaporation. Water and food were not given during the experiment. A crossover design was used to test the effects of the three treatments, viz. normal, dehydrated and starved.

Dehydration. This was always carried out at 21° C. and 55 p.c. relative humidity, water being withdrawn and the only food given being sheep nuts containing 10 p.c. water. Detailed composition has been described previously (Bentley, 1955).

Prevention of licking. The animals were kept in the proximity of the people involved in the experiments for some weeks before the experiments were commenced. They were fitted with a leather collar during this period. The purpose of this treatment was to tame the animal as much as possible so as to minimize excitement during the experiments.

Large 'Elizabethan' type collars 10 inches in diameter were cut out of light transparent plastic. These, when fitted over the heads of the tamed animals, caused no excitement and effectively prevented the animals from licking any part of their body. It should be noted that they also prevented the animal from lying down and assuming the stretched out position so often seen in macropods exposed to high environmental temperatures. Food and water were withdrawn and the cages were raised so that the urine and faeces dropped out of reach of the animals.

Rectal temperatures. These were measured with an electric Universal thermometer type 3 and the appropriate attachments. The readings were to 0·1° C. and the calibration was checked against a standard thermometer.

RESULTS.

Prevention of fur licking. The effect of this treatment on rectal temperature after three hours at 40° C. and 40 p.c. relative humidity was tested. Initial temperatures were not recorded as the crossover design of the experiment allowed for any variation in this. It can be seen from Table 1 that each animal reached a remarkably constant temperature on different days irrespective of whether it wore a collar or not. The differences between the rectal temperatures of the different animals were considerable and were statistically significant. The small difference between the mean rectal temperatures with and without collars was not significant. In the collared animals the fore and hind paws were very wet, obviously not from saliva but from sweat. Bentley (1955) demon-

TABLE 1.

Rectal temperatures in ° C. of Setonyx *with and without fur licking after 3 hr. at 40° C. and 40 p.c. relative humidity.*

Animal	Day 1	Day 2	Day 3	Day 4	Means
1	38·4*	37·9	38·1	38·4*	38·20
2	38·2*	38·2*	38·0	38·1	38·13
3	37·5	37·7*	37·4*	37·4	37·50
4	39·0	39·0	38·8*	39·0*	38·95
Means	38·28	38·20	38·08	38·22	38·20

* Animals with collars (i.e. no fur licking): mean 38·2625
Animals without collars (i.e. normal): mean 38·1250.
Distribution of asterisks shows the orthogonal nature of the data.
The differences between the animals are significant.
($F=74·41$ on 3 and 6 D.F. $P<0·001$).
The effect of collars was not significant ($F=3·99$ on 1 and 6 D.F.).
Error variance from the analysis of variance is 0·018958 (° C.)² on 6 D.F.

TABLE 2.

Evaporative water loss in Setonyx brachyurus *in 6 hr. at 32° C. and 55 p c. relative humidity.*

	Water loss gm.	Difference ± S.E. gm.	P
Normal	30·8	13·3 ± 2·15	<0·01
Dehydrated 4 days	17·5		
		8·2 ± 1·24	<0·01
Starved 3 days	25·2		

The results are the mean values of the same group of 6 animals.
The difference of 5·1 gm. between the normal and starved animals is not statistically significant ($P>0·1$).

strated sweat glands on the pads of the fore and hind paws but nowhere else on the body. In the animals without collars the licking did prevent the sweat welling up in the fur and had a spreading action, but the volume of saliva which became added to the sweat is unknown. However, it was insufficient to reduce the temperature of the animals appreciably.

Evaporative water loss. It can be seen from Table 2 that evaporative water loss was reduced by dehydration for four days. However, it was found that food intake declined sharply when no water was available. In the six animals used in the experiments the mean normal food intake (sheep nuts) was measured on each of three successive days. The mean value was 97 gm. (S.E. ± 3·9) with a daily water intake of 229 ml. (S.E. ± 55·8). On the first day without water the food intake dropped to 61 ± 1·7 gm. and on successive days to 38 ± 1·5 gm., 32 ± 4·4 gm. and 25 ± 4·5 gm. During four days' dehydration, therefore, 156 gm. of food were eaten, which was equivalent only to about 1½ days on normal rations. The effect of three days' starvation on evaporative

water loss was also measured in the same animals. As Table 2 shows, dehydration *per se* reduces evaporative water loss considerably more than does starvation.

Temperature regulation in normal and dehydrated animals. The mean rectal temperatures of ten normal animals kept at 21° C. was found to be 37·42° C. and after four days' dehydration it was 36·19° C. The difference of 1·23° C. is highly significant ($t = 4·270$, $P < 0·01$). In six of the above ten animals rectal temperature was again measured after six hours in a room at 32° C. The increase in rectal temperature was 0·87° C. greater in the dehydrated than in the normal animals. ($t = 2·906$, $P < 0·05$.)

Four of the above ten animals were subjected to a shorter and more intense heating than the others. When exposed to a temperature of 40° C. for three hours, the rectal temperature rose 1·88° C. when they were dehydrated compared to 0·48° C. when the same animals were normally hydrated. The difference of 1·4° C. is statistically significant ($t = 4·947$, $P < 0·02$). The actual rectal temperature reached in the normal animals was 38·10° C. and 38·55° C. in the dehydrated animals. Thus the lower initial rectal temperature in the dehydrated animals partially offset the greater rise on heating so that the final rectal temperature was not very much higher than when they were normally hydrated.

DISCUSSION.

The observations of Bartholomew (1956) and of Robinson and Morrison (1957) that *Setonyx* assisted regulation of its body temperature at high environmental temperatures by salivating on the fur were not confirmed. They made two assumptions which are open to question:

1. That the moisture on the fore and hind limbs was principally saliva and not sweat. No experiments such as those done in this paper were performed to check this and the relative importance of evaporation from the limbs and that from the mouth and respiratory passages was not tested.

2. That the salivation which accompanied high environmental temperatures was a thermoregulatory response and not merely an accompanying event such as a symptom of hyperpyrexia. The suggestion that salivation on the fur is an important method of cooling in marsupials in general should be treated with caution till more conclusive experiments have been performed on a number of species.

Variation in rectal temperatures between different animals was greater than in the same animals with and without collars, and this further emphasizes the lack of any effective selective advantage for such behaviour. Salivation obviously occurs, but the relative contributions of this and sweat to the wetting of the paws is not clear, despite the opinion of Robinson and Morrison (1957) that this moisture is mainly saliva. The arms and feet of *Setonyx* were wet even when licking was prevented. The contribution of evaporation from the limbs of *Setonyx* to temperature regulation is unknown, but panting and insensible

water loss from the skin are probably of primary importance as in many placentals.

Like the camel (Schmidt-Nielsen *et al.*, 1956) and rat (Dicker and Nunn, 1957) *Setonyx* reduces its evaporative water loss when dehydrated and this is correlated with increased changes in the rectal temperature in animals in a hot environment. Insensible water loss is related to the basal metabolic rate (see Rothman, 1954) and it seemed possible that reduced water loss was due to the voluntary starvation observed in dehydrated *Setonyx*. The experiments, however, did not indicate this. Also, dehydration still reduces water loss in the camel which unlike other mammals does not reduce intake of food when dehydrated (Schmidt-Nielsen *et al.*, 1956). Rectal temperature was seen to drop in starved *Setonyx* but decreased even further in dehydrated animals. This may be a reflection of a change in basal metabolic rate. The possible importance of reduced evaporative water loss to marsupials living in hot arid regions is obvious. However, dehydrated animals show a more rapid increase in rectal temperature than do normal animals and thus may well be less able to withstand high temperatures for as long as normal quokkas. Also, dehydration may affect the proximate cause of death on exposure to heat. For example, the depletion of body fluids in the dehydrated animal may predispose to death from circulatory collapse (heat exhaustion), whereas death from heat stroke (hyperpyrexia) may be relatively more frequent in the normally hydrated animal. Thus the survival value of this reduced evaporative loss remains to be assessed and behavioural observations in the natural habitat would be important.

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SERUM PROTEIN LEVELS AND THE "AUTO-IMMUNE" COMPLEMENT-FIXATION REACTION

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SUMMARY.

Sera from 297 patients were tested for the "auto-immune" complement-fixation reaction, and the serum proteins were analysed by electrophoresis and measured. Apart from a slightly increased incidence of hyper-gammaglobulinaemia among the positive reactors, no exclusive or regular correlation between the reaction and the levels of the various fractions was observed.

INTRODUCTION.

Gajdusek (1957, 1958) and Mackay and Gajdusek (1958) have described an "auto-immune" complement-fixation (AICF) reaction between human sera and human organ antigens. The serum activity lay in the gamma-globulin fraction and there are good, but not over-riding, reasons for regarding it as being due to the action of antibody which may have auto-antibody specificity. On the other hand it may be an adventitious reactivity of serum globulins, not antibody-like and not arising as a specific response to tissue antigen. Most of the positive results reported by Gajdusek and Mackay were in patients suffering from illnesses characterised by hyperglobulinaemia.

In order to show whether the "auto-immune" complement-fixation reaction regularly accompanies a rise in the serum globulin levels such as may be observed frequently in hospital cases, we report below the results of AICF testing of the sera of 297 patients whose serum protein fractions were measured after electrophoretic separation.

MATERIALS AND METHODS.

Patients' sera.

The sera were consecutive samples received for serum electrophoresis in the Biochemistry Division of this Institute. They had been sent for this analysis either because they were already known to have a raised total serum protein level, or because the clinician in charge

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of the case suspected a condition such as liver disease, "collagen" disease, nephrosis, macroglobulinaemia or myelomatosis. The number of electrophoretic analyses undertaken each week was limited, and sera were tested in this way only when the nature of the case warranted it. No attempt was made by us to check any of the clinical diagnoses; we were interested only to see whether there was a general relationship between the AICF reaction and an abnormal alteration in any of the serum protein fractions, irrespective of the disease category.

Quantitative estimation of serum proteins by electrophoresis.

The method was based on the well-known technique of Flynn and de Mayo (1951) using a "tent" type of paper-strip electrophoretic tank. In order to obtain accurate and reproducible quantitative results, it was essential to ensure a direct linear relationship between protein concentration and dye bound to protein. This was thoroughly investigated in the Biochemistry Division of this Institute by G. K. Wilkinson who gave a preliminary report on the method to the A.N.Z.A.A.S. meeting in Melbourne in 1955 (Wilkinson and Wilkinson, 1956). The required linear relationship could be obtained if the washing of the dyed papers was performed in such a way that nearly all the dye absorbed in the paper was removed without losing any of the dye bound to the protein. Minute attention to detail was essential during the washing procedure, particularly as regards time of washing and composition of the washing solutions.

Twenty µl. of serum were applied with a graduated pipette along a 2·5 cm. pencil line drawn centrally across the 4·0 cm. paper strip, which gave a free margin of at least 0·5 cm. on each side. It was important to apply the serum evenly so that the separated bands are sharply defined.

The method was standardised and corrected by running pure albumin and globulin solutions concurrently with the sera. Crystalline human albumin and freeze-dried human globulin were obtained from the Commonwealth Serum Laboratories, Melbourne. 10, 20 and 30 µl. of 4·0 p.c. human albumin in 0·9 p.c. sodium chloride (i.e. one-half, the same, and one-and-a-half times the volume of serum applied, which gave, in effect, 2 p.c., 4 p.c. and 6 p.c. solutions on the paper), and 20 µl. of 1·0 p.c. globulin, were applied to paper strips and run at the same time as the sera.

The tank was filled with the buffer solution (barbitone buffer pH 8·6; $\mu = 0{\cdot}06$) and allowed to equilibrate for 30 minutes before the papers were inserted. The prepared strips were carefully wetted with the buffer solution to within 2·0 cm. of the pencil line and placed in position over the centre rod in the tank. Electrophoresis was carried out at 110-120 volts and 1·0 milliamp per strip.

After running for 16 hours, preferably overnight when the ambient temperature was steadier, the strips were dried in a hot air oven not exceeding 105° C. for 30 minutes. The redundant paper was cut off and the strips placed in a flat enamel dish and covered for exactly 10 minutes with a fixing solution of 10 p.c. mercuric chloride in 10 vol. p.c. glacial acetic acid in ethanol. They were then dried again in the same oven, washed with water, once more oven dried, and laid flat in an enamel dish and stained for exactly 10 minutes with a 1·0 p.c. solution of brom-phenol blue in 10 vol. p.c. glacial acetic acid in ethanol. It was advisable to push the strips under the dye solution with a glass rod. The brom-phenol blue solution was poured off and could be used again if it was filtered. 0·5 p.c. acetic acid in 25 p.c. ethanol was then poured over the strips, which were once again pushed under with a glass rod. The dish was gently agitated for 10 minutes. The solution was poured off and replaced with a fresh quantity. This was repeated for three washes of 10 minutes each and then for two washes of 15 minutes. Finally, the papers were again dried in the oven.

(If the papers were to be scanned electrophotometrically for semi-quantitative estimation at this stage, they were held temporarily in a jar containing a small amount of ammonia at

the bottom, without touching the liquid, and then dipped in xylene which made them sufficiently transparent.)

On the serum strips the distance in cm. was measured from the origin to the end of the α_1-globulin, which was the beginning of the albumin fraction. This was done for all the serum strips in the batch and an average calculated. This average distance, which represented the uniform "albumin trail", was marked on the 2 p.c., 4 p.c. and 6 p.c. albumin strips and on the serum strips. Then the leading albumin bands (as distinct from the "trails") both from the standard albumin and from the serum papers were cut off and eluted to 50 ml. with 1·5 p.c. sodium carbonate in 50 p.c. methanol.

The individual globulin fractions, α_1, α_2 and β, were measured in cm. on all the serum strips, the distances were recorded and the papers were cut. Each individual fraction was eluted in 12·5 ml. 1·5 p.c. sodium carbonate in 50 p.c. methanol. The "trails" from the albumin standards were not cut and were eluted in the same way.

All elutions were allowed to stand for approximately one hour. The eluates were mixed gently by inversion and then read in a Unicam SP400 spectrophotometer at 596 mμ using a 1·0 cm. cuvette and a distilled water blank. The percentage transmission readings of the eluates were converted to arbitrary dye units by reference to a previously prepared graph of readings from dilutions of brom-phenol blue dye in 1·5 p.c. sodium carbonate in 50 p.c. methanol, the percentage transmission having been plotted against units of dye.

Another graph was made by plotting units of dye against the concentrations of standard albumin and connecting the points with a straight line. Then the value of the albumin fractions in the unknown sera could be read directly from it.

The uncorrected separate globulin fractions were converted directly from the units of dye taken up by the standard 1·0 p.c. γ-globulin. The eluted standard albumin "trails" were calculated in terms of globulin per cm. for each of the standards. The standard (2, 4 or 6 p.c.) which was nearest to the albumin level of each unknown serum was chosen, and the "trail" value per cm. for that standard was subtracted from each globulin fraction of that serum according to the length in cm. of the fraction. As the γ-globulin fraction rarely overran the origin by more than 1·0 cm. the amount of protein subtracted from this fraction was taken as that contained in 1·0 cm. of albumin trail.

AICF reaction.

This was performed as described by Gajdusek (1958), with some changes in the complement-fixation test, which was a five-volume technique using 0·2 ml. volumes in W.R. tubes. Sera were diluted 1/10 with subsequent doubling dilutions to 1/20, 1/40, 1/80, and so on. Serum dilutions, antigen, and 2½ HD_{50} of complement (previously titrated in the presence of antigen) were allowed to stand overnight at +4° C. Next day after one hour at bench temperature, each tube received two volumes of haemolytic system. The tests were incubated at 37° C. for 30 minutes, and then lightly centrifuged. 50 p.c. haemolysis was taken as the end-point. A "negative result" was failure to fix complement at a serum dilution of 1/10. Each test included a serum anti-complementary control, and a known positive and a negative serum was added to each batch.

RESULTS.

Among the 297 sera there were 54 positive AICF reactors, which is 18·2 p.c. of the series. This is similar to the results obtained in another and larger series of 1014 cases, investigated clinically and serologically, 21·4 p.c. of which were positive AICF reactors (Hackett *et al.*, 1960).

The positive sera fixed complement with one or more of the following antigens: liver, kidney, lung, adrenal, and thyroid. We divided these into 18 *strong positives* showing a titre of 1/80 or greater with one or more of the antigens, and 36 *positives* with titres of less than 1/80. The mean serum protein measurements of each of these two categories were contrasted with those of the *negative* AICF reactors.

Only the albumin and the γ-globulin fractions showed any progressive change in mean serum level from the negative, through the positive, to the strong positive AICF reactors, the albumin level falling and the γ-globulin rising. The α_2 and β-globulins showed slightly lower levels among the strong positives than among the rest. Table 1 summarises these findings.

TABLE 1.

Mean levels of serum proteins (gm. p.c.) in sera of negative, positive, and strong positive AICF reactors among 297 sera.

	Negative 243 cases	Positive 36 cases	Strong positive 18 cases
Total proteins	6·67	6·97	6·93
Albumin	3·68	3·46	3·14
Total globulin	3·42	3·51	3·78
α_1-globulin	0·34	0·35	0·36
α_2-globulin	0·76	0·78	0·70
β-globulin	0·96	0·98	0·91
γ-globulin	1·35	1·37	1·76

DISCUSSION.

The most important reason for considering the γ-globulins as a single group of proteins, despite physical and immunological evidence of heterogeneity among them, is their biological activity as the main antibody fraction of human plasma. Animals reared in a germ-free environment have very marked hypo-gammaglobulinaemia, and it is possible that such γ-globulins as they do produce may be due to stimulation by antigens which it is not possible wholly to eliminate from their food (Gustaffson and Laurell, 1959; Wostmann, 1959). On this basis, all γ-globulins may be antibodies. On the other hand, some normal γ-globulins may not be antibodies, and in diseases such as myelomatosis or macroglobulinaemia the abnormal proteins which appear in the γ-globulin fraction have almost certainly not been produced as a result of ordinary antigenic stimulation.

The causes of the hyper-gammaglobulinaemia which accompanies many non-infectious diseases are not understood. But if AICF activity was an adven-

titious by-product of excess globulin production it might accompany hyperglobulinaemia with some regularity. Our results here show that this is not so. One-quarter of those sera which gave a positive AICF test had a γ-globulin level above 1·60 gm. p.c., but so did one-quarter of the whole series. (The serum albumin shows the reciprocal effect.) The 18 patients who gave a strong positive AICF reaction had a slightly higher mean γ-globulin level (1·76 gm. p.c.) than the 36 weaker positives (1·37 gm. p.c.) or the negatives (1·35 gm. p.c.). On the other hand four of the five sera with a γ-globulin level above 3·0 gm. p.c. had no detectable AICF reaction.

TABLE 2.

Percentages of AICF reactors in sera where the protein levels lie outside the normal range. Percentage of AICF reactors in total of 297 sera = 18·2 p.c.

	Number of sera in the abnormal range out of total of 297 cases	Percentages in the abnormal ranges of			
		negatives	positives	strong positives	positives + strong positives
		p.c.	p.c.	p.c.	p.c.
Albumin <3·80 gm. p.c.	168	78	14	8	22
α_1-globulin >0·45 gm. p.c.	36	75	14	11	25
α_2-globulin >0·80 gm. p.c.	89	80	17	3	20
β-globulin >1·15 gm. p.c.	50	86	12	2	14
γ-globulin >1·60 gm. p.c.	71	75	14	11	25

Taking the limit of lower normality of the albumin as 3·8 gm. p.c., and of upper normality of the globulins as α_1 0·45 gm. p.c., α_2 0·80 gm. p.c., β 1·15 gm. p.c., and γ 1·60 gm. p.c. (Owen, 1958), the proportions of strong positives, positives and negatives among the sera which fell outside these margins are shown in Table 2. It is clear that although the incidence of AICF reactors is slightly raised in all these abnormal categories except among the raised β-globulins, it is so slightly above the average of 18·2 p.c. for all cases in the series as not to be significantly associated with an abnormal rise in any globulin fraction or a fall in albumin.

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THE PERIPHERAL DILATOR ACTION OF RESERPINE IN MAN

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SUMMARY.

Infusion of reserpine into the brachial artery produces a very marked and prolonged vasodilatation which is confined to the skin of the limb. During the dilatation there is no detectable increase in sensitivity to noradrenaline nor any reduction in the response of the vessels to ephedrine or to reflex sympathetic activity. While some degree of noradrenaline depletion of the tissues cannot be excluded, the findings suggest that if such depletion occurs, it must in some way release vascular tone without causing other manifestations of depletion.

INTRODUCTION.

Reserpine, a crystalline alkaloid of *Rauwolfia serpentina*, is widely used in the treatment of hypertension. Its hypotensive effect is usually attributed to its central action in reducing sympathetic vasomotor activity (Chopra, Gupta, Mukherjee, 1933; Trapold, Plummer and Yonkman, 1954; Bein, 1955; McCubbin and Page, 1958). Suggestions to the contrary, however, have been made by Iggo and Vogt (1959) and Brodie, Spector and Shore (1959). The possibility that reserpine may have some local dilator action on peripheral blood vessels is indicated by the observation of McQueen, Doyle and Smirk (1955) that injection into the perfused rabbit hind limb caused a fall in perfusion pressure and is supported by the fact that the drug can cause depletion of catechol amines from peripheral tissues in animals (Bertler, Carlsson and Rosengren, 1956; Muscholl and Vogt, 1958; Burn and Rand, 1958).

This paper reports the effects of locally applied reserpine on the peripheral limb blood vessels in man, central nervous and haemodynamic effects being excluded. The results support the hypothesis that the hypotensive action of reserpine is at least in part a consequence of its local effect on peripheral vessels.

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METHODS.

The subjects were ourselves, our colleagues and volunteer medical students, ages ranging from 19 to 35 years. The experiments were carried out at a laboratory temperature of 23 ± 1° C. The subject lay recumbent on a couch for at least 30 min. before observations began. Blood flow measurements in the forearm or hand of both sides were made using the technique of venous occlusion plethysmography with water-filled plethysmographs at a temperature of 35 or 32° C. respectively. Intra-arterial infusions were given into the brachial artery at the elbow of one side through a needle (21 or 23 S.W.G., 3 cm., short bevel) connected by a length of polythene tubing to one of two mechanically-driven syringes. During control periods 0·9 p.c. NaCl w/v was infused at a constant rate of 4 ml./min. Drug solutions were made up in 0·9 p.c. NaCl so that the dose per minute was contained in 4 ml. and were substituted for the saline solution as required.

In a number of experiments the circulatory changes in the skin and muscle of the forearm were differentiated by measuring the venous oxygen saturation of returning venous blood using a spectrophotometric technique (Roddie, Shepherd and Whelan, 1957a).

Assay of the plasma noradrenaline level of the returning venous blood from the forearm was performed in a number of experiments. Blood samples were taken from a venous catheter into nylon syringes, placed in nylon test tubes kept on ice, and centrifuged at 5° C. The plasma was injected intravenously into the cat pretreated with the combination of substances used by Karki (1956) or into the pithed rat (Shipley and Tilden, 1947). Carotid artery pressure was recorded manometrically.

The drugs employed were reserpine (Serpasil, Ciba), ephedrine hydrochloride (Elliotts and Australian Drug Pty. Ltd.), methylamphetamine hydrochloride (Methedrine, Burroughs Wellcome) and l-noradrenaline bitartrate (Levophed, Winthrop). Doses are expressed as weights of the base in each case.

RESULTS.

Vasodilatation in the upper limb.

Reserpine introduced into the brachial artery in a dose of 50 μg./min. for 10 min. had no effect on the forearm blood flow during the period of infusion. Within 10-20 min. of the end of the infusion the skin of the forearm became flushed and the blood flow began to rise, reaching a maximum in 1-1½ hours (Fig. 1). The blood flow then slowly returned to the previous resting level during the next 24 hours. No such changes occurred in the control forearm, the flow through which remained unchanged throughout. The subject was released from the apparatus after the first three hours of observation and resumed his normal daily activities, returning to the laboratory at varying intervals for the recording of further observations.

The pattern of response described above was seen in 13 experiments on nine subjects in which the forearm blood flow was followed and in five experiments on five subjects on whom the hand blood flow was determined. The skin of the forearm and hand remained flushed and was subjectively warmer throughout the period that the blood flow was elevated.

In two experiments double the dose of reserpine was given in the same time (100 μg./min. for 10 min.). The effects on the forearm and hand blood flow

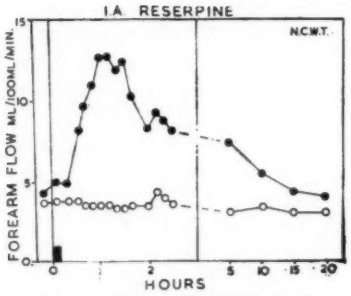

Fig. 1. The response of the forearm blood flow to intra-arterial infusion of 50 μg./min. of reserpine for 10 min., indicated by the black rectangle. • injected side; ○ control side. Flow is expressed as ml. per 100 ml. of forearm tissue per minute. The values for the blood flow before the infusion of reserpine are the mean of the 20 observations recorded during the preceding 5 min. Each subsequent value is the mean of observations recorded at 15 sec. intervals for 2-4 min. The time scale is different in the two halves of the diagram.

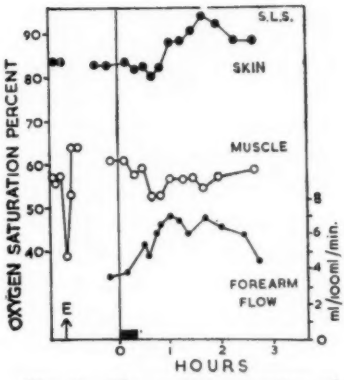

Fig. 2. The oxygen saturation of the venous blood returning from a superficial vein draining skin (•) and a deep vein draining muscle (○) of the left forearm. The forearm muscles were exercised rhythmically for one minute at E. The black rectangle represents the infusion of reserpine (50 μg./min. for 10 min.).

were as described, but in addition there developed nasal congestion, conjunctival injection and postural hypotension of a moderate degree.

Infusion of the reserpine vehicle alone had no effect on limb blood flow.

Skin and muscle vessels.

In six experiments on four subjects the changes in forearm muscle and skin blood flow in response to intra-arterial reserpine were determined by simultaneous measurement of the oxygen saturation of the returning venous blood obtained from nylon catheters introduced into a superficial and a deep vein of the forearm. The data from one of these experiments are shown in Fig. 2. Before injecting the reserpine into the brachial artery the forearm muscles were exercised for one minute. Oxygen saturation of the deep muscle blood immediately fell and then rose above the resting value after the exercise ceased, while the superficial blood saturation showed no increase or a slight fall. This preliminary test demonstrated that the catheters were in fact sampling skin and muscle blood respectively. At the end of the experiment Evans blue dye (500 μg./min.) was infused into the brachial artery for two minutes during which time blood samples were withdrawn simultaneously from the superficial and deep catheters. In each case dye was present in the plasma from both sites, indicating that both catheters were sampling blood draining from areas which were being perfused by the intra-arterial infusion.

In all experiments the oxygen saturation of the superficial blood rose to 95-100 p.c. saturation within 40 minutes of the end of the injection of reserpine, the increase sometimes commencing before any rise in the total forearm blood flow measured by the plethysmograph became detectable, and full saturation was reached before the blood flow had reached its maximum. The blood from the deep catheter, draining mainly muscle, did not show any such increase in saturation, but remained steady throughout. Venous sampling was continued for 1½-2 hours after the end of the infusion of reserpine, by which time the blood flow and the skin oxygen saturation had begun to decline.

Response of reserpine treated vessels to sympathetic activity and to sympathomimetic drugs.

The blood vessels of the reserpine treated limb responded in the normal way to injections of ephedrine and methedrine (Fig. 3), and the hand blood flow showed the usual vasomotor fluctuations even at the height of the reserpine-induced vasodilatation. Reflex vasomotor stimuli such as a loud noise or the application of ice to the head and neck resulted in vasoconstriction of equal degree on both the control and the reserpine treated side (Fig. 4).

The magnitude of the constrictor response of the hand vessels to intra-arterial or intravenous noradrenaline was the same during the reserpine dilatation as before or after (Figs. 4, 5).

There was thus no evidence of increased sensitivity to noradrenaline of vessels treated with reserpine nor was there any diminution of the constrictor responses to ephedrine, methedrine or to sympathetic stimulation. The same findings were obtained in nine experiments on eight subjects.

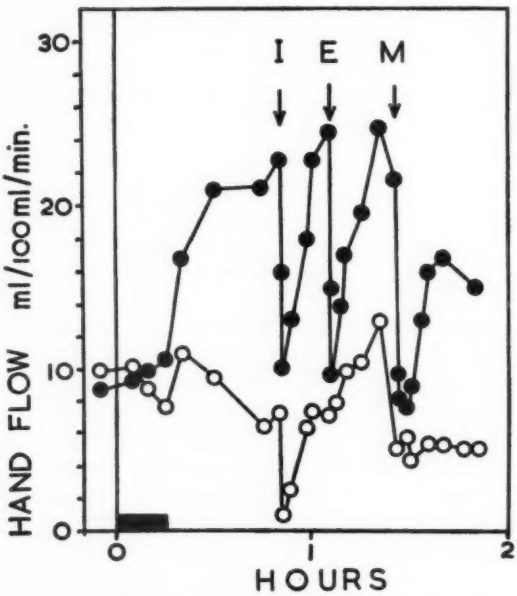

Fig. 3. The response of the hand blood flow to the application of ice to the head (I); to intra-arterial ephedrine 0·25 mg. in 1 min. (E); and methedrine 0·125 mg. in 1 min. (M) during the dilatation following infusion of reserpine into the brachial artery in a dose of 50 µg./min. for 15 min. (black rectangle). • Injected side; ○ control side.

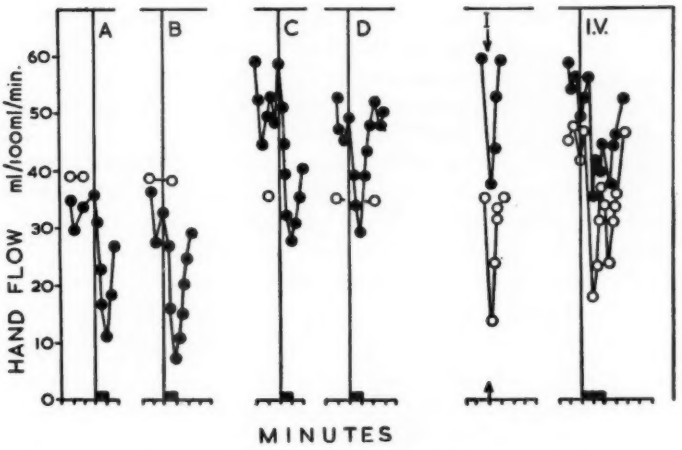

Fig. 4. The response of the hand blood flow to intra-arterial noradrenaline infusions in a dose of 0·2 μg./min. before (A and B) and during (C and D) reserpine vasodilatation. At I ice was applied to the forehead and neck. I.V. indicates an intravenous infusion of noradrenaline (10 μg./min.). • Injected side; ○ control side. The subject was indirectly heated throughout the experiment by immersion of the feet and legs in a water bath at 44° C. to release vasomotor tone.

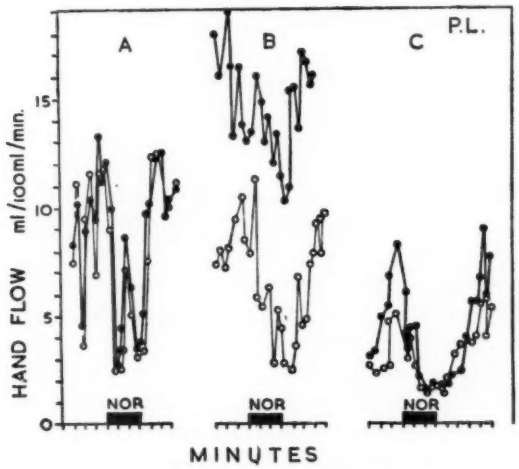

Fig. 5. The effect of intravenous infusions of noradrenaline (10 μg./min.—black rectangles) on the left (•) and right (○) hand blood flow A before, B during, and C after, reserpine dilatation of the left side.

Noradrenaline in blood.

Samples of the returning venous blood from the skin of the forearm were taken during the infusion of reserpine into the brachial artery, in the 10-20 minutes after the infusion when the blood flow was still unchanged, and at intervals during the subsequent dilator phase.

No increase was detected in the plasma noradrenaline level during any of these periods. The method employed did not permit the detection of noradrenaline in concentrations less than 2·5 ng./ml.

DISCUSSION.

The dilator effect of reserpine on the blood vessels of the forearm and hand, when it is infused into the brachial artery in doses which are insufficient to have any general systemic effects and which do not produce effects on the opposite limb, demonstrates that this drug has a local action on peripheral vessels which is independent of any central depression of vasomotor activity. The local blood concentration of the drug in the forearm is unknown, but on the basis of infusion at a rate of 50 μg./min. into an arterial inflow of 5 ml./100 ml. of forearm volume/min. it can be calculated that the local concentration in the arm during the infusion was of the order of 1·5 mg./litre and although this is somewhat greater than the probable blood concentration during reserpine therapy it is likely that the local dilator action of the drug plays a part in its hypotensive action.

A striking feature of the vasodilatation following intra-arterial reserpine was the length of time that it persisted, the blood flow often being still elevated 24 hours after the infusion. Such a prolonged vasodilatation is not seen following intra-arterial injection of other dilator drugs in common use and it is only equalled by the duration of the dilator response following intra-arterial gases, such as oxygen or carbon dioxide (Marshall and Whelan, 1956).

The selective action of reserpine on the skin vessels, the muscle vessels being unaffected, is difficult to explain; but it is in accord with the findings of Bock and Müller (1956) who gave reserpine intravenously in human subjects and observed no change in the heat clearance from the forearm and calf muscle as determined thermoelectrically at a time when the skin clearance was well elevated.

The time course of the vasodilatation in the human limb, comprising a slow onset and a gradual return to the resting level, would be compatible with the release of noradrenaline and its slow re-synthesis in the chromaffin tissue at peripheral vessels. The same order of time course has been demonstrated for the disappearance of noradrenaline after single parenteral injections of reserpine in rat hearts (Paasonen and Krayer, 1958) and peripheral sympathetic ganglia (Muscholl and Vogt, 1958) and also for the onset of sensitivity changes to

adrenaline and noradrenaline after intravenous reserpine in dogs (Schmitt and Schmitt, 1958).

The selective action on skin vessels is consistent with the possibility that the content of chromaffin tissue and of noradrenaline is higher in forearm skin than in muscle and with the observations of differences in the sympathetic innervations of these two vascular beds (Roddie, Shepherd and Whelan, 1957b).

While all the above considerations point to the likelihood of the noradrenaline depleting action of reserpine being responsible for its local vasodilator effect, we were, however, unable to obtain any evidence for noradrenaline depletion comparable to that of Burn and Rand (1959) on animals. There was no increased sensitivity to injected noradrenaline and no reduction of vasoconstrictor responses to sympathetic stimulation or sympathomimetic drugs at the height of the reserpine dilatation. The fact that reserpine resulted in a further increase in flow in a limb whose sympathetic tone had been released by indirect heating (Fig. 5) is further evidence suggesting an action independent of sympathetic nervous activity.

Some degree of noradrenaline depletion of the limb tissues cannot be excluded, but the findings suggest that if such depletion occurs it must in some way release vascular tone without causing other manifestations of depletion. The possibility exists that the stores of noradrenaline in the tissues exert a tonic influence on vessels independently of the activity of the sympathetic nerves. If this were the case, depletion of the peripheral stores alone could cause vasodilatation without interfering with sympathetic activity. More prolonged exposure to reserpine might be required for the sympathetic nerve ends to be depleted, or sympathetic inhibition might be solely a consequence of the central action of reserpine on the vasomotor centre or the ganglia and would only be seen with generalized reserpinisation of the individual.

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LYSOGENY IN *PSEUDOMONAS AERUGINOSA*[1]

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SUMMARY.

An investigation has been made into lysogenicity and pyocinogenicity in *Pseudomonas aeruginosa*. The bacteriophages isolated were classified into groups on their antigenic properties and attempts have been made to correlate biological characteristics with this grouping. Multiple lysogeny in some strains is described, including one strain lysogenic for four unrelated phages.

Certain of the bacterial strains are lysogenic for a phage having characteristics more typical of a virulent than of a temperate bacteriophage and the relationship of such strains to the isolation of virulent phages from natural sources is discussed.

Reference is made to the application of this knowledge of *Pseudomonas* phages to genetic and epidemiological studies with this bacterium.

INTRODUCTION.

The frequency of occurrence in nature of lysogenic strains of *Pseudomonas aeruginosa* has been previously shown (Warner, 1950; Don and van den Ende, 1950; Alfoldi, 1957). This present paper confirms the observations of these workers and describes the isolation and characterisation of a number of phages active on two selected indicator strains of that bacterium that have been used previously for the study of genetic recombination in *Ps. aeruginosa* (Holloway, 1955, 1956). The purpose of this study was to obtain phages to be used in further genetic studies on this bacterium and also to make further observations on the nature of lysogeny in this organism.

MATERIALS AND METHODS.

Bacteriophages. In general, the procedures described by Adams (1950) have been used. Media used were the stock meat infusion agar and broth as prepared in the Bacteriology Department with the addition of M/1000 calcium chloride. Assays of bacteriophage preparations were made using the layer agar method. High titre phage stocks were prepared by the lysis of broth cultures, the layer agar method, or by induction with ultraviolet light, depending in each case on the particular phage.

Bacteria. Procedures used for the growth of bacterial strains were as previously described (Holloway, 1955). The characteristics of strains 1, 2 (previously labelled strain L) and 3 have been given in that paper. The other strains used in this study have been obtained from various Melbourne hospitals.

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Nomenclature. Bacterial strains are referred to numerically and phage strains by capital letters followed by a number. The capital letter refers to the serological type of the phage, the number to the bacterial strain from which it was isolated.

Antiphage sera. These were prepared by repeated injections of phage stocks (10^9-10^{10} particles/ml.) subcutaneously or intravenously into rabbits. Sera were heated at 56° C. for 30 minutes before use.

Turbidity measurements. These were made using a Coleman Model 7 photonephelometer.

RESULTS.

The occurrence of lysogenicity amongst the strains examined.

Isolates of *Ps. aeruginosa* were obtained from various Melbourne hospitals. Cultures obtained from single colony isolates of each strain were used to inoculate tubes of nutrient broth which, after overnight growth, were spotted with a loop on to lawns of each of the two arbitrarily selected indicator strains 1 and 2. Indicator lawns were prepared by the addition of 1 ml. of nutrient broth and two drops of an overnight broth culture of strain 1 or 2, to 2 ml. of nutrient agar with an agar concentration of 1 p.c. and held at 45° C. The mixture was then poured over the surface of a dried nutrient agar plate. After overnight incubation, a zone of lysis around the spot indicated that the strain was either lysogenic or pyocinogenic (Jacob, 1954) for that indicator strain. These two conditions could be distinguished by centrifugation of a broth culture of the strain in question, and spotting two-fold dilutions of the supernatant on to the particular indicator lawn. A lysogenic strain produced discrete plaques, whereas the pyocins caused a confluent zone of lysis over the area of the spot, decreasing in intensity to extinction with higher dilution.

Results obtained with 81 strains of *Ps. aeruginosa* tested in this manner are shown in Table 1. They indicate the prevalence of lysogeny and pyocinogenicity amongst the strains tested, nearly 75 p.c. of the strains possessing one or other of these characteristics. Where a strain was found to be lysogenic for either or both indicator strains, no determinations for pyocinogenicity were made.

TABLE 1.

Distribution of lysogenicity and pyocinogenicity in Pseudomonas aeruginosa.

	No. of strains
Non-lysogenic and non-pyocinogenic for 1 and 2	23
Non-lysogenic for 1 and 2 but pyocinogenic for 1	7
Non-lysogenic for 1 and 2 but pyocinogenic for 2	16
Non-lysogenic for 1 and 2 but pyocinogenic for 1 and 2	15
Lysogenic for 1	7
Lysogenic for 2	9
Lysogenic for 1 and 2	4
	—
	81
	—

Isolation and classification of Pseudomonas *phages.*

The following procedure was used to isolate the phages from the lysogenic culture. Overnight broth cultures of the lysogenic strains were centrifuged and the supernatants treated with chloroform to kill the remaining bacterial cells. The supernatants were then diluted and plated as for an assay of infective particles to obtain single plaques. Single plaque isolations were made and used to prepare high titre stocks of the phage (usually by means of the layer agar technique). These stocks were stored in ampoules in a deep freeze at – 20° C.

Classification of the phages isolated has been based on their antigenic characteristics, a method suggested by Adams (1953). The ability of particular antiphage sera to neutralise the infectivity of phage particles of related types may thus be applied as a simple criterion of identification. Using this test most of the phages isolated to date have been placed in the serological groups described below where each serological group is denoted by a capital letter.

Group A. Phages of this group have a solid centre plaque with an irregular edge. Individual isolates have plaques varying from 1-3 mm. in diameter. Three different types of the group have been found with respect to host range; one type plates on strain 2 only (A77, A74, A52, A53, A101); a second type plates on both indicator strains 1 and 2 (A83, A96, A106), and the third type plates on strain 1 only (A3 and A86). The plaque morphology on strains 1 and 2 is very similar. All these types are inducible by ultraviolet light and this has been found to be the most satisfactory way of producing high titre stocks of this group. Phages belonging to Group A are unstable both at room temperature and in the cold. Clear plaque mutants of this phage have been observed only once and then at low frequency. Group A phages are the most frequently encountered.

Group B. Five isolations of Group B have been made, B3, B110, B127, B134 and B140. On strain 1, the plaque has a diameter of 1-2 mm. with a central area of clear lysis surrounded by a ring of growth, this in turn surrounded by a ring of lysis. Clear plaque mutants of B3 have been isolated. Plaque-forming particles of B3 occur in broth cultures of 3 at a concentration of less than 20 particles/ml. Phage B3, propagated on strain 1, plates with high efficiency on strain 1 but with very much reduced efficiency (10^{-6}) on strain 2. When the plaques formed on strain 2 are propagated on strain 2, the phage so resulting plates with high efficiency on strain 2 but with low efficiency on strain 1. This situation is thus a host-induced modification of the adaptive kind and represents the type of reciprocal modification postulated by Luria (1953).

Group B phages are non-inducible and can transduce using strain 1 as the receptor and donor strain (Holloway and Monk, 1959, and unpublished).

Group C. Only one isolation of this group has been made, from strain 3. Like B3, plaque-forming particles of C3 exist in broth cultures of this strain at

very low titre, generally less than 100 particles/ml. C3 plates on strain 1, but not on strain 2. On strain 1 very small, indistinct plaques are formed. It is not inducible by ultraviolet light. Clear plaque mutants occur at frequencies of about 1 per 5,000 normal plaques. Using strain 1 as the donor and acceptor strain, C3 can transduce auxotrophic markers at low frequencies.

Group D. Six separate isolations of this phage have been made, D3, D100, D110, D141, D281 and Dsew. This last isolate was made from Melbourne sewage and was a clear plaque mutant. The group D phages isolated from 3 and 100 on strain 1 have solid centre plaques, quite circular with an entire edge. There is a very distinct halo extending into the surrounding lawn of bacterial growth. Plaque size is constant at about 2 mm. in diameter. D3 is inducible by ultraviolet light. Clear plaque mutants are observed in platings of this phage at a frequency of about 1 in 500 normal plaques.

Group E. Three representatives of this group have been isolated, E79, E141 and E281. This phage forms clear plaques 1-2 mm. in diameter on strains 1 and 2. It demonstrates a number of interesting characteristics, different from those of the other phages isolated; and its nature and origin are discussed in more detail in the section below on virulent phages.

Group F. Only one phage of this group has been studied. Obtained from strain 116 it plates on both strains 1 and 2 forming circular solid centre plaques 2-3 mm. in diameter. F116 is capable of transduction using either strains 1 or 2 as donor or acceptor.

Other groups. Other phages have been isolated from *Pseudomonas* strains obtained from various hospitals and by reason of their plaque morphology, host range, and antigenic specificity obviously fall into groups other than those characterised above. Further study on these phages will be deferred for the present.

Multilysogenic strains of Pseudomonas.

One feature of the analysis of the lysogenic strains has been the demonstration that a number of these strains are lysogenic for more than one phage. Strains carrying two prophages are quite common. One strain, strain 3, was studied in detail and was found to be lysogenic for four phages, A3, B3, C3 and D3, all serologically dissimilar. A3 and D3 exist in a normal lysogenic state, the titres of infective particles for these phages in a broth culture of strain 3 being about 10^5-10^6. The titres of B3 and C3 on the other hand are extremely low being less than 100 plaque-forming particles per ml. The presence of B3 and C3 was detected by plating culture filtrates of strain 3 on indicator strain 1, artificially made lysogenic for both A3 and D3. There is no prophage cross immunity between A3, B3, C3 and D3. Two suggestions can be put forward to account for this low titre of infective particles for B3 and C3. The low rate of production of mature phage for B3 and C3 could be due to interference with the prophages of A3 and D3 which results in preferential production of the

latter phages. Alternatively B3 and C3 could be defective prophages in strain 3 and the small number of infectious particles found in culture filtrates could represent mutations from the defective to the complete form. Certainly B3 as isolated from strain 3 can lysogenise strain 1 to produce a normal lysogenic strain. The phage titre of a broth culture of strain 1, lysogenic for B3, is 10^6 infective particles per ml. It is curious that B3 and C3 are both competent in transduction, a property not shared by A3 and D3.

The isolation of virulent phages.

One of the purposes of this study was the isolation of phages to enable the production of phage-resistant mutants of strains 1 and 2 thus providing additional markers for recombination experiments. One way of doing this is to look for clear plaque mutants of the lysogenic phages. As has been shown for lambda phage (Kaiser, 1957) and Salmonella phage P22 (Levine, 1957), clear plaque mutants have a reduced lysogenisation frequency. When such mutants are plated with sensitive bacteria so that the phage is in excess, most of the bacteria are lysed leaving only those which, by virtue of a mutation, are unable to absorb the phage, and hence are resistant. Such resistant mutants have been obtained and have been used in genetic studies (Holloway and Fargie, 1960). Some interrelationships between the various groups of phages have been revealed by the study of the resistance patterns. If strain 1 is made resistant to a phage of group E, say E79, then the resistant strain also becomes resistant to A, C and D groups of phages. When bacterial strain 1 is made resistant to D3, three types of resistant mutants may be obtained. The first is completely resistant to both D3 and E79; the second is resistant to D3 but not to E79; the third type of mutant, while not being truly a resistant mutant, results in highly modified plaques when D3 is plated on it.

The nature and origin of E group phages are somewhat of an enigma. As isolated from, say, strain 79, this phage forms clear plaques on both indicator strains 1 and 2. In either solid or liquid cultures it causes complete lysis of these sensitive strains. Furthermore, it differs from all other phages isolated in two characteristics. Firstly, by the effect of both the live virus and the UV-inactivated virus on the optical density of an infected logarithmic culture of a sensitive strain, and secondly, in its sensitivity to ultraviolet light. We shall consider these points in more detail.

Logarithmic phase cultures of either strain 1 or 2 are infected at a multiplicity greater than one. By following the optical density of such infected cultures throughout the latent period a significant difference is revealed between infection with E79 and infection with a temperate phage such as D3 even when clear plaque mutants of the latter are used (to minimise effects of lysogenisation on the optical density). With D3 there is a rise in optical density during the latent period (as shown in Fig. 1), but with E79 there is little, if any, rise during this period. Thus infection with E79 immediately arrests bacterial growth, a property shared with the T-even phages.

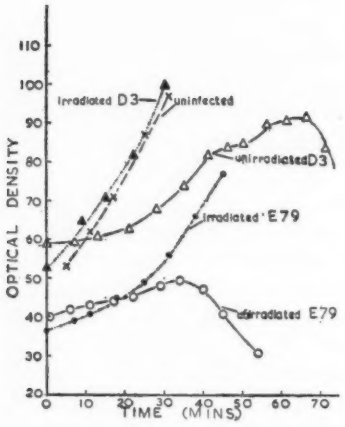

Fig. 1. Changes in optical density when *Ps. aeruginosa* strain 1 is infected separately with UV-radiated and unirradiated phages E79 and D3. The uninfected growth curve is shown as a control.

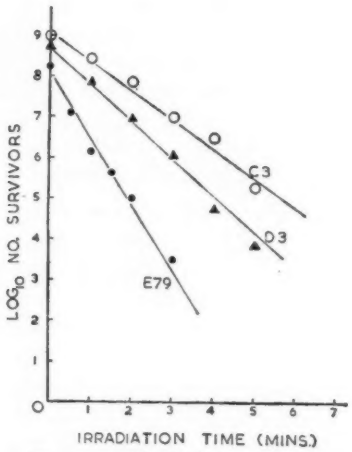

Fig. 2. Ultraviolet inactivation curves of *Pseudomonas* phages C3, D3 and E79.

Furthermore, E79 differs from other *Pseudomonas* phages examined in possessing a higher sensitivity to ultraviolet light. The rate of ultraviolet inactivation was determined for phages E79, D3 and C3, in the following manner. A 15 W General Electric germicidal lamp was used to irradiate 5 ml. of a phage suspension of 5×10^8 plaque forming particles/ml. held in buffer (Krieg, 1959) in a continuously agitated 3½ in. petri dish at a distance of 50 cm. from the UV tube. Precautions were taken to prevent photoreactivation. The UV inactivation curves of E79, C3 and D3 are seen in Fig. 2 and it is clear that E79 is more sensitive to UV than either D3 or C3.

As pointed out by Jacob and Wollman (1959) an important characteristic of temperate phages is their inability when inactivated by ultraviolet light to kill sensitive bacteria on which they are adsorbed. To determine whether this situation holds for the system under discussion, phage suspensions of E79 and the temperate phage D3 in buffer, were irradiated with UV to reduce their infectivity titre from *ca.*10^{10} to less than 10^4 plaque-forming particles/ml. Logarithmic phase cultures of strain 1 were separately infected with unirradiated and irradiated phage suspensions at a multiplicity of 5-10 and changes in the optical density were followed with the nephelometer. The results are shown in Fig. 1. Whereas the growth curve of the culture infected with the UVL inactivated D3 follows the curve of the uninfected strain 1, infection with UVL inactivated E79 markedly reduces the increase in optical density and its action, for

a period almost equivalent to the latent period, is similar to the non-irradiated phage preparation. On the basis of this criterion as proposed by Jacob and Wollman, E79 does not fit the description of a temperate phage and its behaviour is more characteristic of a virulent phage.

Some strains producing E group phages have been found to be unstable for this characteristic and on serial subculture may entirely lose this property.

Pyocinogenicity.

Jacob (1954) has described the identification of pyocinogenic strains of *Ps. aeruginosa* analogous to the colicinogenic strains of Enterobacteriaceae. Pyocinogenicity for the two selected indicator strains is quite common amongst the strains examined in the present study. Apart from the differentiation of pyocinogenicity from lysogenicity and the use to which this may be put for epidemiological studies, no further studies have been made on pyocins.

DISCUSSION.

The initial purpose of this study, the application of bacteriophage techniques to genetic studies with *Ps. aeruginosa,* has been achieved. Phage resistance markers have been successfully used in studies on sexual recombination in this organism and a number of phages competent in general transduction have been demonstrated. The general results described above indicate that not only are lysogenicity and pyocinogenicity of frequent occurrence amongst strains of *Ps. aeruginosa,* but that the phages concerned have properties of special interest. The classification of phages by their antigenic characteristics has been found to be successful in practice and it is hoped to extend the knowledge of the biological characteristics of the phages in relation to their antigenic groupings. Already some correlation has been indicated in that group B phages are generally able to transduce. The frequent occurrence of lysogenicity and pyocinogenicity in *Ps. aeruginosa* has suggested a method of typing strains of this bacterium for epidemiological purposes, analogous to that used for *Salmonella* by Boyd (1950) and Atkinson (1955), and this work is currently in press (Holloway, 1960).

The E group phages have provided further information concerning virulent phages. This term has been somewhat loosely used in the literature to describe a heterogeneous collection of phages having the common property of producing clear plaques. The various types of virulent phages can be listed in the following manner:

1. C-type mutants (Kaiser, 1957; Levine, 1957). These are commonly referred to as weak virulents; they are mutants of temperate phages which have a reduced ability to lysogenise. Such mutants are unable to overcome homologous prophage immunity.

2. Virulent mutants of temperate phages able to overcome prophage immunity. This group would include the 'inducing virulents' of lambda (Jacob and Wollman, 1954).

3. Virulent phages which kill the sensitive cell by the action of a lethal phage protein. This group includes the T-even phages which in addition produce an essential destruction of the bacterial nucleus.

Group E phages give lytic reactions similar to those of type 3 and show some similarities to the T phages. Broth cultures of sensitive bacterial strains are lysed completely with the production of a highly viscous lysate. Sensitive strains do not become lysogenic for phages of this group. The fact that UVL inactivated phages cause a marked reduction in bacterial growth when they infect an exponentially growing sensitive strain suggests that this effect may be similar to the lethal protein action known for certain T phages. The higher sensitivity of E79 to ultraviolet light when compared to the other temperate phages tested does not suggest any host reactivation by the sensitive strain 1. Additional work along both these lines will be needed to substantiate any analogy of the E group phages with the T-even coliphages. While the isolation of a phage with these characteristics is not unusual, we are not aware of any previous demonstration of the production of these phages by a bacterial culture in a manner analogous to that of a temperate phage. Bertani (1958) has suggested that there is no real difference between a virulent and a temperate phage except one of degree and describes the T-even phages as having greater evolutionary specialisation towards parasitism than a temperate phage. The E group phages could represent an intermediate step in this parasitic trend.

The ability to produce a group E phage is unstable in most of the cultures initially having this characteristic. However, the fact that more than 1 p.c. of the strains tested possess this property indicates that the relationship must be more stable under natural conditions than those prevailing in the laboratory. The frequent isolation of virulent phages for a range of bacteria from intestinal contents or sewage may be a reflection of this greater stability in these environments. Appropriate testing of newly isolated strains of enteric bacteria could conceivably produce phages related to the T group. As pointed out by Bertani (1958) the extent of the knowledge of the chemistry and genetics of the T phages makes any information concerning their origin potentially very valuable for studies on the general nature of lysogeny.

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THE UPTAKE OF ISONIAZID AND RELATED COMPOUNDS BY *MYCOBACTERIA*

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SUMMARY.

Other hydrazides inhibited the uptake of isoniazid. If the hydrazide group was substituted there was no inhibition. C^{14} nicotinic acid hydrazide was taken up at approximately one-third of the rate of uptake of isoniazid. The uptake required oxygen and was sensitive to sodium cyanide and azide. In this the uptake resembled that of isoniazid, but there was no inhibition by other hydrazides even in large excess.

Hydrazones of isoniazid were taken up by a different process which did not require oxygen and was insensitive to cyanide.

These results are discussed in terms of the mode of action of isoniazid.

INTRODUCTION.

A large part of the literature devoted to the possible mode of action of isoniazid consists of reports of the inhibitory action of isoniazid on enzyme reactions measured either with cell-free enzyme preparations or in whole cells. Difficulties of interpretation have always prevented any definite conclusions from being drawn. Some of the difficulties involved in trying to correlate biochemical tests with bacteriological observations have been described elsewhere (Youatt, 1958a) and the present paper is specifically concerned with the difference that exists between isoniazid and its isomers, nicotinic and picolinic acid hydrazides. An attempt has already been made to use this difference in the interpretation of biochemical tests. Hicks and Cymerman-Craig (1957) showed that transaminase activity was inhibited equally well by the three isomers and deduced that this could not be the normal site of action of isoniazid unless a permeability barrier existed in *Mycobacteria* for the other hydrazides.

If the possibility of a permeability barrier could be removed then the comparison of isoniazid inhibition with that produced by other hydrazides would be a useful experimental approach. It would be expected that the enzyme which was the site of action of isoniazid would be considerably less sensitive to nicotinic and picolinic acid hydrazides than to isoniazid.

When this work was begun, since no other radioactive hydrazide was available, an indirect approach was used. This was to measure the uptake of radioactive isoniazid in the presence of other unlabelled hydrazides on the assumption that if they were also taken up they would compete for the same transfer

mechanism. Later, radioactive nicotinic acid hydrazide was prepared and direct measurements were made.

It was previously suggested that the mechanism of transfer of hydrazones of isoniazid into the cells was different from that of isoniazid. The work reported here confirms this view.

METHODS.

Cultures. *Mycobacterium tuberculosis* (B.C.G.) was grown on the surface of a modified Sauton medium, harvested, washed and suspended in phosphate buffer for the measurement of radioactive uptake with isoniazid. Full details of all the procedures have been described (Youatt, 1958a).

Radioactive materials. C^{14} isoniazid, labelled in the carbonyl group, was obtained from the Radiochemical Centre, Amersham, England. The specific activity was 2 mc./mM. The hydrazones of isoniazid were prepared by heating together in alcoholic solution equimolar amounts of the aldehyde and the C^{14} isoniazid, then evaporating to dryness. The residue was extracted with ethyl acetate, which was then evaporated off. Solutions were made up in phosphate buffer pH 7 and no isoniazid was detectable chemically.

C^{14} nicotinic acid was obtained from Amersham. This was diluted with C^{12} nicotinic acid to give a specific activity of 2 mc/mM and the hydrazide was prepared by the method which Murray and Langham used for the preparation of isoniazid (1952). The sample obtained melted at 160° C. and did not depress the melting point of a sample of C^{12} nicotinic acid hydrazide. A stock solution was prepared and this gave the theoretical radioactive count and the colour estimation with picryl chloride was in complete agreement.

Other reagents. Dr. Cymerman-Craig of the University of Sydney kindly supplied the following reagents: nicotinic acid hydrazide, m.p. 160·5-161·5° C.; picolinic acid hydrazide, m.p. 100° C.; benzoic acid hydrazide, m.p. 112-113° C.; 1-*iso*nicotinyl-1-methyl hydrazide-2HCl, m.p. 221° C. and *iso*nicotinic acid hydrazide methiodide, m.p. 210-211° C. Verazide, m.p. 187° C. was supplied by Mr. Vaughan of this department. Cyanacetic acid hydrazide was supplied by Professor A. Albert of the Australian National University, Canberra.

RESULTS.

Fig. 1 shows the uptake of C^{14} isoniazid in the presence of equimolar amounts of benzoic, nicotinic and picolinic acid hydrazides. Cyanacetic acid

TABLE 1.

Uptake of isoniazid in the presence of related compounds.

Isoniazid-sensitive B.C.G. suspensions in phosphate buffer pH 7 with 40, 30, 20 or 10 μg/ml. of isoniazid and 20 μg./ml. of the related compound. Both compounds were added simultaneously and the suspensions were shaken in air at 35° C. for 30 minutes. The rate of uptake was determined by radioactive count. Results are expressed as μg. isoniazid per gm. dry cells. A. verazide; B. 1-*iso*nicotinyl 1-methyl hydrazine dihydrochloride; C. *iso*nicotinic acid hydrazide methiodide.

	μg. isoniazid per gm. dry cells. Isoniazid concentration μg./ml.			
	40	30	20	10
Control	66	57	41	19
Control	62	57	35	20
A.	74	50	31	15
B.	59	50	34	18
C.	70	58	42	16

hydrazide was also found to inhibit isoniazid uptake. These results indicated that the $-NH\text{-}NH_2$ group was important and that it was immaterial whether it was attached to a pyridine ring, a benzene ring or a straight chain.

Table 1 shows the uptake of isoniazid in the presence of two compounds in which the $-NH\text{-}NH_2$ group has been substituted. Both *iso*nicotinic acid hydrazide methiodide and nicotinamide failed to inhibit the uptake of isoniazid. This further indicates the importance of the hydrazide group.

An attempt was made to determine whether the inhibition of isoniazid uptake by other hydrazides was competitive. Data are shown in Table 2. The results were plotted as 1/V against 1/S where V = the rate of uptake of isoniazid and S = the concentration of isoniazid, but it was apparent that the processes involved were too complicated for this simple treatment. The hydrazides are not only taken up but are metabolised (Youatt, in press) and the acids may also be able to enter into other metabolic pathways.

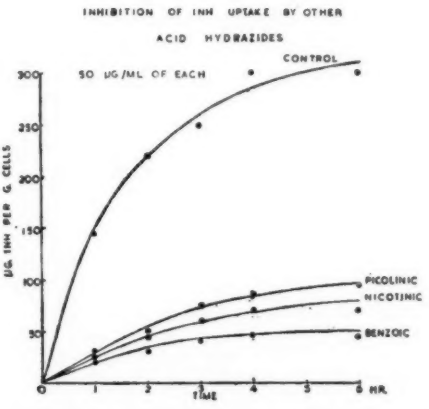

Fig. 1. *The inhibition of isoniazid uptake by other hydrazides.* Isoniazid-sensitive B.C.G. suspensions in phosphate buffer pH 7 were shaken in air at 35° C. with radioactive isoniazid and equimolar amounts of other unlabelled hydrazides. The rate of uptake, expressed as µg. isoniazid per gm. dry cells, was determined from the radioactive count.

The uptake of C^{14} nicotinic acid hydrazide. Nicotinic acid hydrazide was taken up by *Mycobacteria* under the same experimental conditions as were used for the uptake of isoniazid (Youatt, 1958a). In order to determine whether a

TABLE 2.

Inhibition of isoniazid uptake by other hydrazides.

Isoniazid-sensitive B.C.G. suspensions in phosphate buffer pH 7 with 40, 30, 20 or 10 µg./ml. isoniazid and 20 µg./ml. of other hydrazides. The hydrazides were added simultaneously and the suspensions were shaken in air at 35° C. for 30 minutes. The rate of uptake was determined by radioactive count. Results are expressed as µg. isoniazid per gm. dry weight of cells.

	µg. isoniazid per gm. dry cells. Isoniazid concentration µg./ml.			
	40	30	20	10
Control	76	65	36	20
Control	76	58	36	23
Benzoic acid hydrazide	31	18	8·4	3·4
Nicotinic acid hydrazide	38	27	14	7·3
Picolinic acid hydrazide	46	31	25	6·2

similar process was involved, the uptake of nicotinic acid hydrazide was measured in the absence of oxygen and in the presence of sodium cyanide and sodium azide. As with the uptake of isoniazid, oxygen was required and the process was sensitive to cyanide and azide. Results are shown in Table 3.

TABLE 3.

Uptake of radioactive nicotinic acid hydrazide.

Sensitive B.C.G. suspensions in phosphate buffer pH 7 were shaken in air at 35° C. with 20 μg./ml. nicotinic acid hydrazide. Results are quoted in μg. hydrazide per gm. dry weight of cells taken up per hour.

Conditions	Rate
Viable, shaken cells	46
Killed, shaken cells	7·2
Viable, unshaken cells	2·3
Viable, shaken cells with NaCN $M \times 10^{-3}$	6·9
Viable, shaken cells with NaN_3 $M \times 10^{-2}$	5·8

The rates of uptake of isoniazid and nicotinic acid hydrazide by the same cell suspensions were compared and in each case the rate of uptake of isoniazid was greater. A few examples are shown in Table 4.

TABLE 4.

Comparison of the rates of uptake of radioactive isoniazid and nicotinic acid hydrazide.

Sensitive B.C.G. suspensions in buffer pH 7 were shaken in air at 35° C. with 20 μg./ml. of the hydrazides. Results are quoted as μg. hydrazide per gm. dry weight of cells taken up per hour. Each pair of results was obtained with a different batch of cells.

Isoniazid	Nicotinic acid hydrazide
45	14
60	17
35	13

Since other hydrazides inhibited the uptake of isoniazid it was of interest to see what effect isoniazid and other hydrazides had on the uptake of nicotinic

TABLE 5.

Uptake of radioactive nicotinic acid hydrazide in the presence of other hydrazides.

Isoniazid-sensitive B.C.G. suspensions in phosphate buffer pH 7 were shaken in air at 35° C. with 40 μg./ml. radioactive nicotinic acid hydrazide and 2,000 μg./ml. of isoniazid (INH), benzoic acid hydrazide (BH) and picolinic acid hydrazide (PH). The rates of uptake of nicotinic acid hydrazide are quoted as μg. per gm. dry weight of cells per hour.

Conditions	Rate
Control	25
with INH	20
with BH	19
with PH	19

acid hydrazide. Equimolar concentrations of other hydrazides did not inhibit the uptake of nicotinic acid hydrazide and Table 5 shows that even in the presence of 50-fold excess of these hydrazides there was little evidence of inhibition.

Table 6 shows the uptake of C^{14} isoniazid and C^{14} nicotinic acid hydrazide simultaneously. The combined uptake was equal to the sum of the rates of each C^{14} compound in the presence of the other unlabelled hydrazide.

TABLE 6.

Uptake of isoniazid and nicotinic acid hydrazide in the presence of each other.

Isoniazid-sensitive B.C.G. in phosphate buffer pH 7 shaken in air at 35° C. The rates were determined by radioactive count and are quoted in μg. of the hydrazide per gm. dry weight of cells per hour.

Conditions	Rate
20 μg./ml. C^{14} INH	73
20 μg./ml. C^{14} INH + 40 μg./ml. C^{12} NH	29
20 μg./ml. C^{14} INH + 40 μg./ml. C^{14} NH	68
40 μg./ml. C^{14} NH	37
40 μg./ml. C^{14} NH + 20 μg./ml. C^{12} INH	41

Uptake of nicotinic acid hydrazide by cultures. Nicotinic acid hydrazide was taken up by growing B.C.G. cultures. With 0·5 μg./ml. isoniazid added to the medium beneath a Sauton surface culture, the organisms sank within 24 hours and the radioactive count was equivalent to 10·4 μg. isoniazid bound per gm. dry weight of cells. Under the same conditions with the addition of nicotinic acid hydrazide, the cells continued to grow in the normal way and the radioactive count at 24 hours was equivalent to 5·8 μg. nicotinic acid hydrazide bound per gm. dry weight. In cultures dispersed with Tween 80 the cells exposed to nicotinic acid hydrazide bound more radioactivity than the cells with isoniazid. It is not known why this difference exists between surface and dispersed cultures unless it be assumed that not all the organisms of a surface mat are in contact with the medium.

TABLE 7.

Uptake of radioactive isoniazid, verazide and phtivazid.

Isoniazid-sensitive and resistant B.C.G. suspensions in phosphate buffer pH 7 were shaken in air at 35° C. for 2 hours with 10 μg./ml. of isoniazid and the equivalent amounts of the hydrazones. The results are expressed as μg. isoniazid per gm. dry cells so that they may be directly compared.

	μg. isoniazid per gm. dry cells		
	INH sensitive viable	INH resistant viable	INH resistant killed
Isoniazid	81	7	14
Verazide	40	43	48
Phtivazid	31	25	38

Hydrazones of isoniazid. It was previously reported that pyridoxal *iso*nicotinoyl hydrazone was taken up equally well by living and dead cells (Youatt, 1958a). The same observation has now been made with the *iso*nicotinoyl hydrazones of veratric aldehyde (verazide) and vanillin (phtivazid).

The failure of verazide to inhibit the uptake of isoniazid was further evidence that its mechanism of transfer was different (Table 1). Further, the uptake of verazide was not inhibited by sodium cyanide which inhibited the uptake of isoniazid and nicotinic acid hydrazide.

DISCUSSION.

In seeking the mode of action of isoniazid the comparison of isoniazid-sensitive and resistant cells gives one approach and the comparison of isoniazid with its isomers gives another. The present paper was mainly concerned with determining whether other hydrazides were able to enter the cell. The results show that they do. The direct evidence for this is the radioactive binding by cells exposed to C^{14} nicotinic acid hydrazide which is confirmed indirectly by the inhibition of isoniazid uptake by these other hydrazides.

The requirement of oxygen and the inhibition by cyanide of the uptake of nicotinic acid hydrazide show that the transfer mechanism is similar to that by which isoniazid enters the cell. However, while isoniazid uptake was inhibited by nicotinic acid hydrazide and other hydrazides, nicotinic acid hydrazide uptake was not inhibited by any of these even in large excess. It is not clear whether there is more than one transfer mechanism, one of which is shared by isoniazid or whether there is more than one site of binding.

It seems highly likely that there are different sites of binding at the hydrazide concentrations used in these experiments. It is suggested that at concentrations above 10 μg./ml. hydrazine and all hydrazides share the same mode of action, or what is more likely, modes of action. This might be described as the area of non-specific inhibition. Only isoniazid inhibits in the region of 0·1 μg./ml. This is the specific area of inhibition where there is one particular metabolic process which is sensitive specifically to isoniazid. Since it has been shown that nicotinic acid hydrazide can enter the cell it would appear that the action of isoniazid depends on its stereochemical configuration rather than on its character as a hydrazide.

The difference in the transfer mechanism of the hydrazones of isoniazid is of interest for several reasons. It explains the occasional high rates of uptake by resistant B.C.G. after some hours of exposure to isoniazid (Youatt, 1958a). It has since been observed (unpublished data) that in some cell suspensions isoniazid in the supernatants is wholly bound as a hydrazone after a few hours of incubation. The nature of this hydrazone is not yet known, but it seems likely that it will be the hydrazone of a keto acid. Once formed in the supernatant the hydrazone would be taken up by resistant cells.

The uptake of hydrazones of isoniazid is also of interest because the isoniazid-resistant cells show cross-resistance to the hydrazones. Resistant cells also break down verazide (Youatt, in press). This raises the question whether resistant cells lack the metabolic process which is sensitive to isoniazid or whether resistance depends on a quantitative relationship between the rates of uptake and breakdown of isoniazid and its hydrazones. It has been shown that resistant cells metabolise isoniazid at rates similar to sensitive cells but that they bind radioactivity at much slower rates when exposed to C^{14} isoniazid. It has also been observed with resistant cells that only *iso*nicotinic acid can be detected in the cells (unpublished data). The pigment production which is associated with the action of isoniazid on sensitive cells has never been observed with resistant cells even at high levels of isoniazid (Youatt, 1958b). This last observation suggests that the sensitive metabolic process may be absent from resistant organisms, but it is still possible that the quantitative differences in the rates of uptake and breakdown of isoniazid might supply the explanation of resistance.

It was also considered that the difference between isoniazid and nicotinic acid hydrazide might be explained if the cells broke down nicotinic acid hydrazide as fast as they took it up, but this was not supported by the experimental evidence. Table 4 showed that nicotinic acid hydrazide was taken up at approximately one-third of the rate of isoniazid uptake but that it was also broken down at approximately one-third of the rate (Youatt, in press).

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THE ANTISTAPHYLOCOCCAL ACTIVITY OF HUMAN SERA *IN VITRO* AND ITS RELATIONSHIP TO PASSIVE PROTECTIVE POTENCY

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SUMMARY.

The degree of bactericidal or bacteriostatic effect of human sera against coagulase producing staphylococci was found to vary according to strain and serum. The nature of the antibacterial agent is unknown.

The ability of human sera to protect mice against the lethal effect of intraperitoneally injected living staphylococci of a certain strain (strain Smith) in mucin suspension appeared independent of the effect of the sera against the same strain *in vitro*.

In a preparation of human gamma globulin, the mouse protective factor appeared to have been concentrated; divergent results were obtained in two kinds of test for antibacterial activity *in vitro*.

INTRODUCTION.

M. W. Fisher (1959) reported that some human sera protected mice against lethal intraperitoneal infection with staphylococci of a certain strain (strain Smith) suspended in mucin. It is known that human sera possess antistaphylococcal properties *in vitro*; the subject has been reviewed recently by Skarnes and Watson (1957). The work reported here was undertaken to examine the relationship between the bactericidal and bacteriostatic potency of human sera to coagulase producing staphylococci and the power of the sera to protect mice passively against a type of experimental disease.

MATERIALS AND METHODS.

Strains. Six strains of coagulase producing staphylococci were used.

1. Smith: this strain was isolated by Dubos in 1930 (Hunt and Moses, 1958); its properties have been described by Smith and Dubos (1956), Finkelstein and Sulkin (1958), Hunt and Moses (1958) and Fisher (1959).*

2. 7530: a phage type 80 strain, isolated in Sydney in 1954 from a pustule in a baby and maintained in the lyophilized state until received in this laboratory.

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* Dr. Phyllis Rountree has found this strain untypable by the standard typing set of staphylococcal bacteriophages. According to the descriptions of Finkelstein and Sulkin (1958), and Hunt and Moses (1958), it consists of two variants one of which is susceptible to lysis by phages 44A and 42E, and the other is untypable.

3. Wood 46: an old strain used widely for the production of supernatant rich in alpha haemolysin.

4. L125-2: isolated from bovine mastitis in Brisbane in early 1959.

5. 96: a strain recently isolated from a healing incisional wound of a case of osteomyelitis of the femur following the plating of a fracture.

6. 91: a strain recently isolated from a case of furunculosis of long duration.

The strains were maintained on nutrient agar slopes under paraffin oil at room temperature.

Sera. Twenty samples of fresh blood were obtained from Red Cross donors; the sera from some of these were pooled (Pool 1 and Pool 2) as described below. One individual serum (F) was obtained from a volunteer who was available for repeated bleeding. Serum P was obtained from a patient suffering from boils. Mouse blood was collected into a pool from normal adult male animals by severing the axillary vessels under ether anaesthesia. All samples of blood were allowed to clot, the sera separated aseptically on the day of collection and preserved in the deep freeze. The gamma globulin used had been prepared at the Commonwealth Serum Laboratories (Melbourne) from pooled normal human serum and contained no preservative.

Cultures, suspensions and viable counts. Subcultures from the paraffin covered slopes were made in nutrient broth every 2-3 weeks and thence to nutrient agar slopes as required for use; the slopes were incubated at 37° C. for seven hours or overnight. Standard suspensions of the growth were made in saline to approximately 10^9 organisms per ml., using Brown's turbidity tubes (Burroughs Wellcome), diluted in saline in serial tenfold or hundredfold steps to 10^{-6}, and the viable units counted by the surface drop method of Miles and Misra (1938) on nutrient agar plates, when the suspensions were to be used for tests *in vitro*. The suspensions were used immediately after preparation.

Bactericidal and bacteriostatic tests. Two kinds of test were performed. In one, duplicate sets of 10 drop volumes of the sera were inoculated with one drop bacterial suspension, using 10^{-6}, 10^{-4} and 10^{-2} dilutions of standard suspension. In the other, duplicate or triplicate sets of 10 drop volumes of threefold serial saline or broth dilutions of the sera were inoculated with a drop of a 10^{-5} dilution of the standard bacterial suspension.

The preparations were made in ¼ oz. screw-cap bottles or 4 in. × ½ in. cotton wool plugged test tubes. The former were stood in holes of a tilted turntable which was rotated at 16 r.p.m. during incubation; the latter were placed in a test tube rack. Incubation was carried out at 37° C. for 15-16 hours. No difference was detected between the results of the agitated and still cultures.

After incubation, viable counts were performed on the cultures and, where necessary, their serial hundredfold dilutions in saline, by the surface drop count method of Miles and Misra (1938). From the means of the colony counts per drop, the numbers of viable units in the 11 drop volumes contained in each culture were computed.

Controls were set up to check the sterility of the sera and broth used. Growth control cultures for the broth dilution tests were also set up; they contained broth with the inoculum used in the test and with 1/10 that inoculum.

Mice. 5-6-week-old white mice of either sex were used, from a random bred colony, the breeders of which were obtained from the Division of Animal Genetics, C.S.I.R.O., Sydney.

Passive immunization and challenge. In most experiments, subcutaneous injection of 0·2 ml. serum over the back of the animal was followed by intraperitoneal challenge about 24 hours later. In one experiment, the intraperitoneal challenge was administered first and 0·2 ml. serum injected by the same route one half-hour later (Fisher, 1959).

Strain Smith was used for challenge. For the immunized groups and the main group of controls, one volume of a 10^{-4} saline dilution of the standard suspension was mixed with ten volumes of 5 p.c. mucin. To determine the number of lethal doses contained in this

mixture, groups of unimmunized mice were also challenged with mixtures of ten parts of mucin and one part of standard bacterial suspension diluted in saline 10^{-5} and 10^{-6}.

The suspension of mucin (hog gastric mucin, type 1701W) was made up according to the instructions of the manufacturer (Wilson Laboratories, Chicago, Ill., U.S.A.).

Most deaths occurred within 24 hours of the injection of the challenge dose; the animals were observed for seven days following the challenge.

Absorption of serum with zymosan. 12 mg. zymosan (Nutritional Biochemicals), spun out of 5 ml. veronal buffer (Pillemer, Blum, Lepow, Wurz and Todd, 1956) was suspended in 6 ml. serum and incubated at 37° C. for one hour, then centrifuged out (5° C. and 6,000 r.p.m. for 15 minutes). The supernatant was absorbed with a second lot of sedimented zymosan at 37° C. for one hour. The mixture was then spun in a Spinco centrifuge (5-10° C., 25,000 r.p.m.) for 1½ hours. The supernatant was separated aseptically and stored in the deep freeze.

As a control for the above, a sample of the same serum was put through the same processes of incubation and centrifugation, but without the addition of zymosan.

Complement titrations. Four drop volumes of undiluted serum and of ½, ¼, 1/6, 1/8, 1/12, 1/18, 1/25, 1/35 and 1/50 serum dilutions in veronal buffer (Pillemer *et al.*, 1956) were mixed with equal volumes of a 1/100 saline dilution of commercial (Commonwealth Serum Laboratories, Melbourne) sheep cell haemolysin and of a 1 p.c. saline suspension of washed sheep red cells. Haemolysis was read visually after incubation for a half hour at 37° C.

RESULTS.

Preparation of serum pools. Aliquots of 20 undiluted sera were screened for their antistaphylococcal activity *in vitro*, against strain Smith, using an inoculum of 560 viable units. Viable counts after incubation were as follows: No growth, 10 sera; 11-100, 2 sera; 110-1000, 4 sera; and confluent growth in all drop platings, 4 sera.

Approximately equal amounts of the eight sera in which the viable count exceeded 110 were mixed to form Pool 1, and the ten bactericidal specimens were mixed in Pool 2.

Fate of staphylococci in undiluted sera (Table 1). All human sera showed some degree of bactericidal or bacteriostatic effect. While Pool 2 was bactericidal to strain Smith, both unabsorbed and absorbed with zymosan, Pool 1 was only moderately bacteriostatic to the same strain. Variations between bactericidal effect and moderate bacteriostasis were also found when different strains were cultured in the same serum (F). Gamma globulin was bactericidal to strain Smith, but less so than Pool 2. Pooled mouse serum was less bacteriostatic than the human sera; from the largest inoculum, strain Smith multiplied to the density of a fully-grown broth culture.

The degrees of bactericidal or bacteriostatic effects varied according to strain and serum. For the same combination, the degree of rise or fall in viable count during incubation tended to be constant and independent of the size of the inoculum; the behaviour of disinfectant agents, in this respect, is said to vary (see Wilson and Miles, 1955a).

TABLE 1.

Fate of staphylococci in undiluted sera.

Serum	Strain	Means of viable counts					
		Inoculum	Incubated preparation	Inoculum	Incubated preparation	Inoculum	Incubated preparation
Pool 1	Smith	7·6	3	$7·6\times10^{3}$	$9·9\times10^{3}$	$7·6\times10^{4}$	$2·8\times10^{4}$
Pool 2	Smith	7·6	0	$7·6\times10^{3}$	0	$7·6\times10^{4}$	9·9
Pool 2	Smith	8·6	0	$8·6\times10^{3}$	0	$8·6\times10^{4}$	$3·1\times10^{3}$
Pool 2 absorbed with zymosan	Smith	8·6	0	$8·6\times10^{3}$	0	$8·6\times10^{4}$	$1·5\times10^{3}$
F	Smith	9·3	5×10^{3}	$9·3\times10^{3}$	10^{4}	$9·3\times10^{4}$	$2·5\times10^{5}$
F	96	4	5×10	4×10^{3}	$5·2\times10^{3}$	4×10^{4}	$1·6\times10^{5}$
F	91	$1·3\times10$	$2·9\times10^{3}$	$1·3\times10^{3}$	$1·2\times10^{6}$	$1·3\times10^{5}$	$2·8\times10^{6}$
F	7530	3·7	3·5	$4·7\times10^{3}$	2×10^{3}	$4·7\times10^{4}$	$1·9\times10^{5}$
F	Wood 46	$1·2\times10$	0	$1·2\times10^{3}$	$5·4\times10$	$1·2\times10^{5}$	$3·3\times10^{3}$
F	L125-2	4·8	0	$4·8\times10^{3}$	$6·8\times10$	$4·8\times10^{4}$	$1·9\times10^{3}$
Gamma globulin	Smith	4·6	0	$4·6\times10^{3}$	$2·8\times10$	$4·6\times10^{4}$	$1·4\times10^{3}$
Pooled mouse serum	Smith	5·2	$1·5\times10^{5}$	$5·2\times10^{3}$	$3·1\times10^{7}$	$5·2\times10^{4}$	$1·3\times10^{8}$

10 drops of serum were inoculated with one drop of bacterial suspension in saline.
Overnight incubation at 37° C.

TABLE 2.

Fate of staphylococci in diluted sera.

Serum	Strain	Diluent	Means of viable counts							
				Incubated preparation						
				Dilution of serum						
			Inoculum	1/3	1/9	1/27	1/81	1/243	1/729	1/2187
Pool 1	Smith	Saline	$4\cdot8\times10$	$4\cdot9\times10^3$	$7\cdot2\times10^3$	$2\cdot1\times10^3$				
Pool 2	Smith	Saline	$4\cdot8\times10$	0	$1\cdot6\times10^2$	$3\cdot1\times10^2$				
Pool 2 absorbed with zymosan	Smith	Saline	$4\cdot8\times10$	0	$4\cdot6\times10$	$1\cdot5\times10^2$				
Pool 1	Smith	Nutrient broth	$4\cdot8\times10$	$5\cdot5\times10^6$	$8\cdot1\times10^7$	H.G.				
Pool 2	Smith	Nutrient broth	$4\cdot8\times10$	0	$6\cdot5\times10^4$	H.G.				
Pool 2 absorbed with zymosan	Smith	Nutrient broth	$4\cdot8\times10$	0	$1\cdot6\times10^6$	H.G.				
P	Smith	Nutrient broth	10	0	0	0	$6\cdot8\times10^6$	$2\cdot7\times10^7$	H.G.	
Gamma globulin	Smith	Nutrient broth	$2\cdot7\times10$	$2\cdot9\times10$	$4\cdot7\times10$	$7\cdot1\times10^3$	$2\cdot7\times10^4$	$2\cdot4\times10^6$	$6\cdot6\times10^7$	H.G.
Pooled mouse serum	Smith	Nutrient broth	$5\cdot2\times10$	$1\cdot6\times10^6$	H.G.					

10 drops serum dilution were inoculated with 1 drop bacterial suspension in saline.
Overnight incubation at 37° C.
H.G.=Culture heavily grown. Viable count not performed.

Fate of staphylococci in diluted sera (Table 2). Organisms of strain Smith multiplied to a moderate extent in 1/3, 1/9 and 1/27 saline dilutions of Pool 1. Pool 2, whether unabsorbed or absorbed with zymosan, was bactericidal to strain Smith when diluted 1/3 in saline or broth; some multiplication occurred in the 1/9 and 1/27 saline dilutions. Diluted 1/27 in broth, neither pool was inhibitory; in the other broth dilutions, Pool 1 appeared about three times less inhibitory than Pool 2. Serum P was the most inhibitory of the human sera tested; it was bactericidal to a dilution of 1/27 and three further threefold dilution steps were required to eliminate the bacteriostatic effect. Gamma globulin was not bactericidal; in consecutive dilution steps the reduction in bacteriostasis was less than in the case of the unconcentrated sera and the effect disappeared in a dilution of 1/2187. The pooled mouse serum was not bacteriostatic beyond a dilution of 1/3.

The saline dilution test on Pool 2 indicated that the decline in the numbers of viable organisms in this serum was due to an antibacterial agent and not to the lack of nutrients.

Mouse protection experiments (Table 3). Pools 1 and 2 did not differ appreciably in protective power; dilutions between 1/3 and 1/27 covered the range from 90-100 p.c. survivals to 90-100 p.c. deaths, the serum being given subcutaneously on the day preceding the challenge. A similar degree of protection was obtained from about 1/10th the amount of serum if it was injected half hour after the challenge, and by the same route; although direct contact between bacteria and serum may have occurred, the two pools showed no difference in protective potency when tested by this technique.

Absorption with zymosan removed a little of the protective substance. The combined survival rates in the groups given the 1/9 and 1/27 dilutions (11/18 and 4/19 for the unabsorbed and absorbed serum respectively) were significantly different ($\chi^2 = 6{\cdot}1$, $0{\cdot}02 > P > 0{\cdot}01$).

The protective power of serum P was very slight; only 20 p.c. of the mice given 1/3 dilution survived.

The protective potency of gamma globulin was about 20 times that of the pooled sera.

The virulence of the challenge suspension was consistent in different experiments, at the approximate level of 100 LD50's. Viable counts on suspensions of strain Smith (the challenge strain), prepared for use in test tube experiments, showed that the LD50 contained approximately 20 viable units; this was contained in 1 ml. mucin suspension. Hunt and Moses (1958) estimated the LD50 of the same strain at 580 cells; their challenge dose was made up in 0·5 ml. mucin suspension. Demonstration of protection by human sera and gamma globulin against mucin suspensions of strain Smith given intraperitoneally is in accordance with the results of Fisher (1959).

Effect of absorption with zymosan on the haemolytic activity of complement in the serum. The titre of Pool 2 was 1/18; after absorption, no haemolytic effect was detectable.

TABLE 3.

Mouse protection experiments.

Serum	Dilution	Survivors/total			
		Immunized	Controls	Titration of 1/10	Challenge dose 1/100
Pool 1	1/3	10/10			
Pool 1	1/9	7/10			
Pool 1	1/27	1/10	0/10	0/5	0/5
Pool 2	1/3	10/10			
Pool 2	1/9	3/10			
Pool 2	1/27	0/10			
Pool 1*	1/10	9/10			
Pool 1	1/30	8/10			
Pool 1	1/100	5/10	0/10	0/5	2/5
Pool 2	1/10	10/10			
Pool 2	1/30	7/10			
Pool 2	1/100	5/10			
Pool 2	1/3	10/10			
Pool 2	1/9	3/10			
Pool 2	1/27	1/9	0/10	0/5	3/5
Pool 2 absorbed with zymosan	1/3	9/10			
	1/9	6/9			
	1/27	5/9			
Pool 1	1/3	7/10			
Pool 1	1/30	1/10	0/10	1/5	1/5
P	1/3	2/10			
P	1/10	0/10			
Pool 1	1/3	9/10			
Pool 1	1/30	4/10	0/10	1/5	3/5
Gamma globulin	1/5	10/10			
Gamma globulin	1/50	9/10			
Gamma globulin	1/500	3/10			

* In this experiment, 0·2 ml. serum dilution was injected intraperitoneally ½ hour after the challenge; in all others, the serum was given subcutaneously on the day before.

For the challenge, organisms of strain Smith, 100,000 per ml. (1 vol.) and 5 p.c. mucin (10 vols.) were mixed. For the challenge titration, suspensions containing 10,000 and 1,000 bacteria per ml. respectively, were mixed with mucin.

The challenge dose was 1 ml.

DISCUSSION.

When tested *in vitro* undiluted, Pool 2 was bactericidal, and Pool 1 only moderately bacteriostatic; in dilution tests, Pool 2 was about three times more bacteriostatic than Pool 1. However, the two pools did not differ in their ability to protect mice. Serum P was more strongly bactericidal and bacteriostatic than Pool 2, but its mouse protective power was lower. The same challenge strain was used in all these tests.

Thus, there appeared to be no correlation between the ability of the sera

to protect mice against the challenge used, and their ability to kill, or to restrict the multiplication of, organisms of the same strain *in vitro*.

In gamma globulin, the mouse protective agent seemed to have been concentrated. Divergent results were obtained *in vitro*. Undiluted, the preparation was bactericidal to the same degree as some unconcentrated human sera; in broth dilution tests the endpoint suggested that the bacteriostatic agent had been concentrated to the same degree as the mouse protective agent. The reason for these findings is not clear.

No attempt has been made to identify the nature of the bactericidal and bacteriostatic agent in human sera against staphylococci. The suggestion of Pettersson (1936) that the agent is a partly heat-stable protein ("beta-lysin") does not appear to have been followed up by chemical studies. Gram positive cocci are not susceptible to the direct killing action of antibody and complement (Wilson and Miles, 1955b). Pillemer, Blum, Lepow, Ross, Todd and Wardlaw (1954) found that a strain of *Staph. albus* was not inhibited by properdin; the zymosan absorption experiments described above indicated that properdin was not concerned in the bactericidal effect of a pool of human serum against the coagulase producing strain Smith. The possible role of coagulase in resisting the antistaphylococcal effect of human serum has been the subject of several studies (Ekstedt and Nungester, 1955; Ekstedt, 1956; Myrvik, 1956; Wlodarczak and Jeljaszewicz, 1959).

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THE EFFECT OF DIET ON ACETATE TOLERANCE IN SHEEP

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SUMMARY.

The rate of disappearance of injected acetate from the vascular system of sheep fed lucerne chaff is much more rapid than that of sheep fed wheaten chaff.

Animals feeding on green pasture respond to injected acetate in a manner similar to those eating lucerne chaff, whereas animals on dry pasture respond like those on wheaten chaff.

Within two days of changing from a diet of wheaten to lucerne chaff the tolerance to acetate characteristic of a lucerne chaff diet can be observed. A longer time is required for the alteration in tolerance when sheep which had been eating lucerne chaff are changed to wheaten chaff.

Possible factors involved in the nature of the diet and their effect on the ability of the sheep to utilize acetate are discussed.

INTRODUCTION.

The previous nutritional history of a monogastric animal is known to affect its ability to utilize carbohydrate as shown by the characteristics of a glucose tolerance curve when an animal has been maintained on a diet rich in fat (Himsworth, 1935; Samuels, Gilmore and Reinecke, 1948).

In ruminants, however, under normal feeding conditions exogenous carbohydrate is not available for metabolic purposes as these animals rely mainly on the lower fatty acids produced in the rumen for their energy requirements. As the amounts and relative proportions of two of these fatty acids – acetic and propionic – can vary widely with different types of fodder (Gray, Pilgrim and Weller, 1951; Reid, Hogan and Briggs, 1957) it was considered that alterations in diet may affect the utilization of these substances by sheep.

Previous work had shown that the determination of an acetate tolerance curve after an intravenous injection of acetate provides a means of assessing an animal's ability to deal with acetate (Smyth, 1947; Jarrett, Potter and Filsell, 1952; Ciaranfi and Fonnesu, 1954). Consequently, a series of observations was made on the tolerance of sheep to acetate when fed on either lucerne chaff or wheaten-hay chaff and when grazing on green or dry pasture. While these

studies were being made, Reid (1958) had reported similar work in which dietary effects on the utilization of acetate and glucose were observed in sheep fed on varying mixtures of wheaten chaff, lucerne chaff and maize.

METHODS.

During September-October, 1956, the tolerance to injected acetate was determined in four merino wethers (age 2-3 years), which were confined in pens and fed a daily ration of 800 gm. wheaten chaff. The ration was then changed to 800 gm. lucerne chaff and the tolerance again determined two months later. The tolerance to acetate was also determined in four wethers (2-4 years) while grazing during the spring months on a mixed pasture and the observations repeated during February, 1957, when the pasture was mature and dry.

To eliminate any possible difference due to seasonal effects another series of observations was made during 1958 on two groups of five merino wethers which had been selected for comparable body weight (30-40 kg.), temperament and feeding habits and which were maintained in pens under identical conditions. Five of the animals received a daily ration of 800 gm. wheaten chaff and the others 800 gm. of lucerne chaff. When a tolerance was being determined, food was withheld until the conclusion of the test. The animals receiving wheaten chaff were drenched with seven mg. cobalt chloride once a week to ensure an adequate supply of vitamin B_{12}.

After the animals had been on this ration for six weeks an acetate tolerance curve was determined by injecting into the jugular vein 5 mM/kg. of acetic acid neutralized to pH 7·0 with sodium hydroxide and diluted to a volume of 100 ml. A pre-injection sample of blood was obtained, the injection given over a period of 2-3 minutes and then blood samples taken at 30, 60 and 120 minutes after the injection. The volatile fatty acids were determined by electrometric titration on a distillate from a protein-free filtrate of the blood (Jarrett, Potter and Filsell, 1952). At the end of the experiment four animals from each group were killed and the concentration of glycogen determined in portions of the liver. Two samples in juxtaposition were obtained from the parietal lobe and two from the ventral lobe by puncturing the liver with a cork-borer and extruding the plug (0·5 gm.) into ice-cold 5 p.c. trichloracetic acid. Samples were obtained within 1-2 minutes after severing the neck and cervical cord, and the glycogen determined by the method of Carroll, Longley and Roe (1956).

Six animals were also used to determine the tolerance to injected acetate during the period immediately following a change in diet. Lucerne chaff or wheaten chaff was fed for several weeks, the acetate tolerance curve established and the ration was changed to wheaten chaff or lucerne chaff respectively and the tolerance again determined on alternate days for several days. Four other animals were similarly treated, but the tolerance tests were not done at such regular intervals. These animals were also given fodder obtained from an area about 60 miles north of Adelaide to eliminate any local qualitative differences.

RESULTS.

Both the 1956 and the 1958 series (Tables 1 and 2 respectively) indicate that sheep fed on lucerne chaff can remove injected acetate from the vascular system more rapidly than sheep fed on wheaten chaff. The mean values for the concentration of volatile fatty acids in the blood at 30 minutes and 60 minutes after an injection of 5 mM of acetate/kg. body weight were 8·4 and 3·7 mM/l. respectively in a group of sheep fed on wheaten chaff and 3·0 and 0·6 mM/l. respectively in sheep fed on lucerne chaff.

TABLE 1.

Concentration of volatile fatty acids in blood (mM/l.)

Lucerne chaff				Wheaten hay chaff			
	Minutes after acetate injection				Minutes after acetate injection		
Animal	0	30	60	Animal	0	30	60
42	0·08	2·70	0·10	42	1·91	8·10	3·40
44	1·10	3·60	0·70	44	0·11	8·21	3·10
80	0·70	3·90	0·70	80	0·13	8·12	3·41
81	0·80	3·40	0·70	81	0·91	9·20	4·70
Mean	0·67	3·40	0·55	Mean	0·76	8·41	3·65
S.E. (mean)		±0·26	±0·27	S.E. (mean)		±0·26	±0·27

TABLE 2.

Concentration of volatile fatty acids in blood (mM/l.)

Lucerne chaff				Wheaten hay chaff			
	Minutes after acetate injection				Minutes after acetate injection		
Animal	0	30	60	Animal	0	30	60
43	0·32	3·97	0·35	45	0·21	7·45	3·30
54	0·69	4·21	0·73	12	0·88	7·08	3·05
73	0·73	4·30	0·70	31	0·85	6·57	2·29
89	0·18	4·00	0·80	50	0·53	6·30	1·63
96	0·39	5·30	0·88	67	0·92	5·80	2·43
Mean	0·46	4·36	0·69	Mean	0·68	6·64	2·53
S.E. (mean)		±0·27	±0·22	S.E. (mean)		±0·27	±0·22

Separate analyses of variance were carried out on the data obtained in 1956 and 1958 (Tables 1 and 2). Significant differences ($P < \cdot001$) between the mean values for wheaten chaff and lucerne chaff were present at both the thirty-minute and sixty-minute periods after an acetate injection in both series.

The difference in acetate tolerance of animals fed on green or dry pasture can be seen in the results shown in Table 3. Significant differences between the mean values of animals feeding on green and dry pasture were present at thirty minutes ($P < \cdot05$) and sixty minutes ($P < \cdot01$). It is apparent that sheep grazing on green pasture respond to an injection of acetate in a similar manner to animals on lucerne chaff, whereas the tolerance of sheep on dry pasture is more like that of animals eating wheaten chaff.

TABLE 3.

Concentration of volatile fatty acids in blood (mM/l.)

Green pasture				Dry pasture			
	Minutes after acetate injection				Minutes after acetate injection		
Animal	0	30	60	Animal	0	30	60
29	0·40	3·01	0·30	19	0·40	8·01	3·22
39	0·30	4·50	0·30	40	0·50	7·75	3·30
47	0·10	6·90	1·20	68	0·88	6·63	1·80
86	0·30	2·10	0·70	70	1·44	8·90	4·70
Mean	0·28	4·13	0·63	Mean	0·80	7·82	3·26
S.E. (mean)		±0·81	±0·44	S.E. (mean)		±0·81	±0·44

Increasing the amount of protein in the diet of those animals on wheaten chaff by adding 100 gm. gluten to the daily ration made no difference to the rate of disappearance of injected acetate. Results are shown in Table 4.

The results obtained from animals maintained on one type of fodder and then changed to the other type indicate that a fairly rapid adjustment takes place. Within two days those sheep which had been changed to lucerne chaff exhibited a tolerance characteristic of this latter diet. After an animal had been on lucerne chaff and then changed to wheaten chaff, however, the change in tolerance was slower. The means of results obtained with three animals in each group are shown in Table 5 and indicate the time lag in response to a change in diet. Some factor implicated in the more rapid rate of removal characteristic of lucerne appears to persist for several days after a change to wheaten chaff. Even after ten days on wheaten chaff the slower rate of removal of

TABLE 4.

Concentration of volatile fatty acids in blood (mM/l.)

Wheaten chaff				Wheaten chaff+gluten			
	Minutes after acetate injection				Minutes after acetate injection		
Animal	0	30	60	Animal	0	30	60
14	0·19	6·81	2·01	21	0·20	8·29	2·75
26	0·10	6·98	1·76	30	0·10	6·79	0·80
14	0·84	8·08	1·55	21	0·43	6·30	3·02
26	0·61	8·24	3·13	30	0·46	6·80	2·50
Mean	0·43	7·53	2·11	Mean	0·30	7·05	2·27
S.E. (mean)		±0·40	±0·43	S.E. (mean)		±0·40	±0·43

TABLE 5.

Concentration of volatile fatty acids in blood (mM/l.)

Mean values for 3 animals in each group with S.E. of means at 30 and 60 minutes = ±0·39 (n′=3, n=36 degrees of freedom).

Days on new diet	Previous diet wheaten chaff changed to lucerne chaff			Previous diet lucerne chaff changed to wheaten chaff		
	Minutes after acetate injection			Minutes after acetate injection		
	0	30	60	0	30	60
0	0·54	6·93	2·85	0·28	3·97	0·33
2	0·35	5·65	1·40	0·34	4·12	0·64
4	0·10	4·95	0·40	0·50	4·89	1·36
6	0·15	3·89	0·67	0·55	4·64	1·24
10				0·46	5·80	1·44

acetate by animals which had been eating wheaten chaff for longer periods was not observed. This trend was also shown in four other animals which were injected with acetate at varying intervals of time after changing the diet.

When both wheaten chaff and lucerne chaff obtained from two widely separated localities were fed to sheep the same major difference in response to acetate was evident between the two types of fodder. There were, however, small differences in tolerance when comparing the two batches of lucerne chaff. These differences were of the same order as those observed between the 1956 and 1958 series as shown in Tables 1 and 2.

The concentration of glycogen in samples taken from the livers of sheep fed lucerne chaff and wheaten chaff showed no significant difference between the two groups; the mean values for each group were 3·1 and 2·7 gm./100 gm. liver respectively (S.E. of mean = 0·30).

In order to test an effect of parenterally administered propionate superimposed on a wheaten chaff diet, three animals received a continuous intravenous infusion of propionic acid neutralized to pH 7·0 with sodium hydroxide. Two animals receiving 70 gm./day in a litre of fluid for three days and one animal receiving 100 gm./day for 10 days showed no significant difference in their ability to remove injected acetate from the blood stream. The propionate infusion was stopped 2-3 hours before the tolerance test was done. The concentrations of volatile fatty acids in the blood of the three animals at 30 and 60 minutes after the acetate injection were 7·1, 6·1 and 8·0 mM/l. and 2·4, 2·3 and 2·5 mM/l. respectively.

DISCUSSION.

It is apparent that the nature of the fodder can materially influence the ability of a sheep to remove injected acetate from the blood stream judged by

the characteristics of an acetate tolerance test. Reid (1958) has made a thorough study of intravenous glucose and acetate tolerance tests in sheep and has reported that not only can the rate of disappearance of injected acetate be considerably altered by diet, particularly when maize is incorporated in the ration, but that tolerance to injected glucose is also affected in adult sheep under these conditions.

While it is thus well established that a sheep feeding on lucerne chaff can deal with injected acetate better than one feeding on wheaten chaff the results reported here give little indication of the mechanism involved. It would appear from the observations of Reid (1958) that the effect may be considered as an enhanced rate of disappearance over a more basal rate rather than any delayed effect due to wheaten chaff. That the former may be the case is suggested by the different time lag in response following a change of diet from wheaten to lucerne chaff and *vice versa*. It is obvious that some factor present in lucerne chaff or actively growing pasture is involved and whatever its nature may be it is likely that it affects the metabolic activity of the animal whereby the effect is sustained for some days after the removal of its source.

In view of the variations in the relative proportions of acetic and propionic acids produced by different diets (Gray, Pilgrim and Weller, 1951; Reid, Hogan and Briggs, 1957) it is of interest to note that little variation in liver glycogen could be detected in the two diets used. It would seem that any effect relating to glycogenesis from propionate could have little if any direct implication.

However, it has been suggested (Jarrett, Potter and Filsell, 1952) that the metabolism of propionate may well influence the subsequent metabolic pathway of acetate and recent work of Pennington (1957) and Felts, Masoro, Panagos and Rapport (1958) on *in vitro* inhibition of hepatic acetate oxidation by short chain fatty acids indicates the need for further investigations into the metabolic interrelationship of these products of digestion in the ruminant. Both Reid (1958) and Shaw (1959) have discussed the importance of the relative proportions of the end products of digestion, but in view of the failure reported here of large amounts of infused propionate to alter the acetate tolerance in animals on wheaten chaff a direct implication of propionate *per se* seems unlikely.

The inability of gluten, when added to a wheaten chaff diet to effect any change in tolerance is interesting in view of its effect on animals receiving small amounts of fluoroacetate. Jarrett and Packham (1956) showed that the addition of 100 gm. of gluten to 800 gm. of wheaten chaff prevented toxic symptoms occurring in sheep when 0·04 mg./kg. of sodium fluoroacetate was administered daily for 40-50 days. This reduced toxicity was also achieved by feeding sheep on lucerne chaff, whereas in sheep fed wheaten chaff toxic effects appeared in ten days.

Following the early work of Himsworth (1935), Reinecke, Ball and Samuels (1939) and Roberts and Samuels (1949) on various metabolic aspects of dietary changes there is now a large amount of evidence to indicate that alterations in

enzyme systems can occur with varying intake of carbohydrate and fat (Freedland and Harper, 1957; Tepperman and Tepperman, 1958). Gallagher and Buttery (1959) have reported that sheep tissues in general have a much lower enzyme activity than rat tissue, particularly in relation to liver glucokinase. Their observations indicate that liver metabolism tends towards formation of glucose rather than its breakdown and that this may be of significance in relation to the dependence of sheep on the oxidation of fatty acids.

The possibility that some factor associated with the digestion of lucerne chaff or green pasture may affect the metabolic activity of the liver and thus influence the utilization of acetate is worth investigating.

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AN APPARATUS FOR THE CONTINUOUS MEASUREMENT OF EATING, DRINKING AND SPONTANEOUS ACTIVITY OF RATS

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SUMMARY.

An apparatus is described which permits consistent and sequential analysis of the general activity, fluid and food intake of rats in psychopharmacological research. The experimental results obtained from the use of this apparatus indicated that methyl phenidate tends to produce significantly higher levels of activity during those periods of the day when these animals are normally quiescent.

INTRODUCTION.

For the purpose of establishing the nature of the relationship that exists between spontaneous eating, drinking, and general activity under varying experimental conditions, it was found necessary to develop apparatus for their accurate quantitative measurement. Furthermore, it was decided to develop the apparatus in such a way that these measures could be obtained for discrete intervals, over any period of time required. Consequently, the apparatus to be described provides a variable quantitative frequency distribution of these activities for any given duration. By suitable separation of the recording unit, it has been possible to isolate the experimental cage and animal in an ideal and undisturbed environment.

METHODS.

The main factor in the design of this apparatus was the necessity of obtaining a quantitative distribution of changes from three intrinsically different information channels. The apparatus consists of three information units as shown in the block diagram in Fig. 1. The activity unit employs as sensory elements two electromagnetic induction coils, with permanent magnets on a moving platform and two fixed coils on the cage assembly. The change in flux produced by the rat's movements induces a small E.M.F. in the fixed coils. This signal is subsequently utilized as a direct measure of spontaneous activity.

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The inherent electrical resistance of the rat, by the action of licking a water nozzle, is used to operate a sensitive relay valve in the drinking unit.

The eating unit is brought into action when the rat opens a sprung, hinged perspex door. The movement of the door triggers the release of a food pellet from a feeder unit, simultaneously giving a temporal signal which is recorded. The three information units were independently built into a 19-inch movable rack, for convenient checking and isolation from the experimental room.

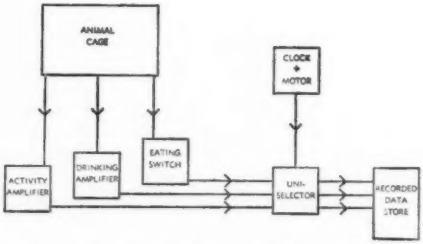

Fig. 1. Block diagram of apparatus.

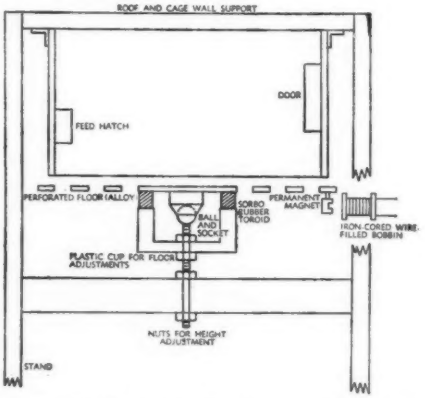

Fig. 2. Diagrammatic view of activity cage.

The animal cage.

The activity cage is an upright cylindrical shape with a perforated disc floor supported by a central ball joint and socket. The walls of the cage are suspended just clear of the floor from the cage assembly (Fig. 2). The floor transmits the changing distribution of the rat's weight, in the form of small angular changes in the horizontal plane of the plate from its normal self-centring position. This in turn produces a change in magnetic flux between the movable magnets and fixed coils. The movements of the floor are dampened and self-centred by a sponge rubber toroid mounted on a plastic cup, centrally disposed beneath the plate. Into the cage wall are admitted the drinking nozzles and the door of the feeder unit. The cage is built into a slotted right angle strip metal frame on castors.

Activity unit.

The signals are generated from two coils disposed at 90° from the centre of the cage on the frame opposite two fixed permanent magnets attached to the floor of the cage. The voltage output of the signals generated are proportional to the rate of induced angular displacement of the magnets. For small movements, imperceptible to the observer, the output voltages are in the region of 50 to 80 mV. For vigorous movements, in the region of 1 to 2 mm. angular displacement, the output voltages rise to several hundred mV. The sensitivity of the fixed coil is a function of the magnetic gap allowed between the coil and the permanent magnet. Using an oscillographic bench display it was possible to determine the relationship between the magnetic gap and the sensitivity range of the coil to varying degrees of acceleration. This relationship has been plotted in Fig. 3. The induced voltage obtained by a given acceleration for a fixed gap of 3 mm. has been plotted in Fig. 4. These values indicate a linear relationship between acceleration (or movement) and voltage generated

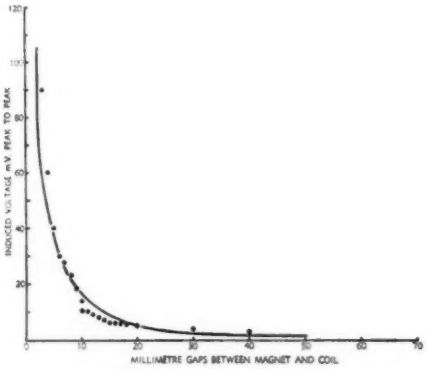

Fig. 3. Induced voltages as a function of the variation of magnetic gap with fixed acceleration.

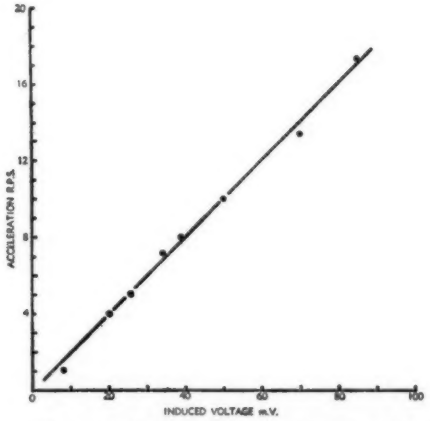

Fig. 4. Induced voltages as a function of the variation of acceleration with fixed magnetic gap of 3 mm.

On the apparatus under discussion the magnetic gap was 3 mm. In practice, however, should there be any undue bias in any plane of movement this can be accommodated. By appropriately adjusting the magnetic gap, the sensitivity of the biased coil can be raised or lowered in that plane without affecting the sensitivity of the other coil. It was found necessary to amplify the small signal generated, because the residual noise was found to be approaching in amplitude the information signals. This was achieved by placing a transistor amplifier adjacent to the floor coil. This yielded a stage gain of 8-10. The amplified signal was fed to a remote unit for further amplification and shaping (Fig. 5).

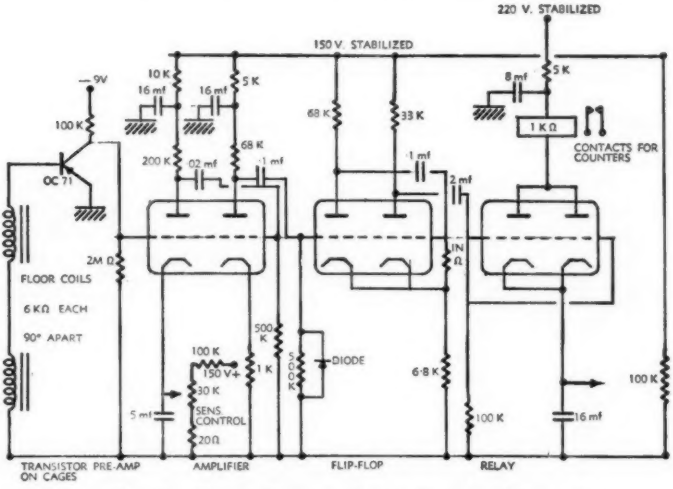

Fig. 5. Circuit diagram of cage floor amplifier unit.

Cage floor amplifier unit.

Stage 1. *Amplifier.* This stage is a double triode with a sensitivity control on the first cathode (pre-set). This gives straightforward amplification which is fed to stage 2.

Stage 2. *Flip flop.* This is a common cathode flip flop wave shaper feeding a large rectangular pulse to stage 3.

Stage 3. *Relay.* This is a further double triode with all electrodes paralleled, driving a Post Office type 3000 relay in the anode with contacts feeding the activity data store via uniselectors. This stage has a pre-set control for setting relay working conditions.

Drinking amplifier.

This unit was designed to determine the temporal distribution of drinking. It was arranged to time the duration of licking from the nozzle of a water bottle. It was established that the maximum speed of licking is about 10 licks per second, consequently the circuit (Fig. 6) was designed to operate at this frequency. It can be seen that this circuit

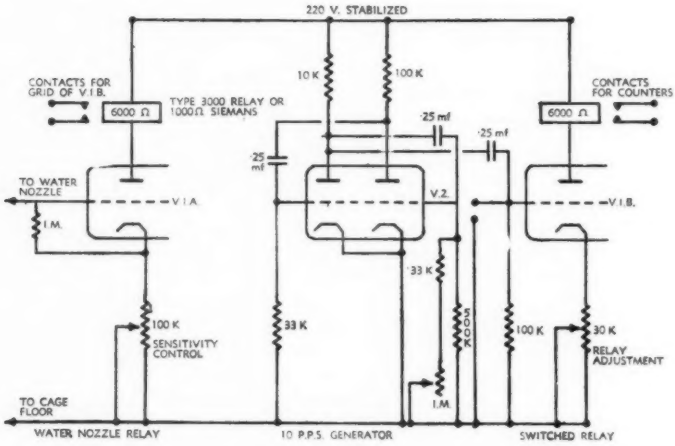

Fig. 6. Circuit diagram of drinking amplifier.

consists of a relay stage in which a relay valve operates when the rat's ohmic resistance is placed from the grid electrode to earth. The current passed by the rat was in the region of 5-15 μA.; this current was well below shock threshold, but sufficient to alter the bias on the valve relay. When the valve relay is tripped a further circuit is brought into operation, generating pulses at 10 per second which are fed to the data store by a uniselector. The overall sensitivity of the drinking relay stage can be varied either to accommodate different degrees of humidity or desired levels of sensitivity.

Eating switch.

This was designed simply to record the operation of opening a hatch door. The contacts on the door are arranged to make a 24 volt circuit, which operates the data recorder via the uniselectors directly. At the same time, the current may be used to operate an automatic feeder apparatus designed to deliver a pellet of food.

Uniselectors.

The purpose of this stage in the treatment of the signals, obtained from the three information channels described above, was to give them a temporal distribution. The operation of the uniselector is by a predetermined timing device. The uniselectors used were arranged as single pole (wiper) 25-way switches; the twenty-fifth contact was used to automatically re-cycle all wipers to position one, on all the banks. All uniselector motors were joined in parallel for synchronous operation.

Mains clock.

For the purpose of providing different temporal distributions for the information received, it was necessary to incorporate a clock with a wide range of contact periods. This consisted of a 50-cycle synchronous clock motor driving a series of cams which directly operate micro-switches. In the experiment to be discussed in the latter part of this paper, the temporal distributions incorporated into the clock were ¼, ½, 1 and 2 hours. The make contacts, in the appropriate micro-switch selected by the experimenter, activate 24 volt motor coils in all uniselectors. Consequently, if a temporal distribution of information was desired using, for example, quarter-hour periods, the appropriate adjustment on the clock unit would ensure that the data were fed via the uniselectors to discrete sections of the record store quarter-hourly.

Data store.

The recorded data store was designed to accommodate a frequency distribution of information from three channels. For this purpose a series of fast R.A.F. fuel flow counters was employed. These were tested for maximum reliable speeds, and were found acceptable when operating at above 10 counts per second. These counters were built up into banks supported on movable 19-inch racks and as independent units were isolated from the experimental environment. Under working conditions it was possible to record a frequency distribution of information from the three channels for a period of 24 hours using, for example, quarter-hour class intervals. This data could then be scored and the store reset for continuous operation.

EXPERIMENTAL.

The experimental animal was placed in the cage and was permitted an extended period of adjustment to the new environment. The cage assembly was placed in a quiet, thermostatically-controlled room; both the amplifiers and the record data store were taken to a neighbouring room. During this settling-in period the rat rapidly learnt to open the food hatch and to locate the water nozzle. When the rat was placed in the apparatus, high initial bursts of activity were recorded, but characteristic rhythms of spontaneous activity, eating and drinking appeared after two to three days. These rhythms appeared to be reasonably stable, but by inspection would have seemed to be rather characteristic of a given animal than of the species generally. For this reason the experimental method adopted when testing rats was to use the animal as its own control. This methodology is particularly suited for the investigation of the effects of small drug doses on the innate eating, drinking and spontaneous activity cycle.

Recent investigations (Glow, in preparation) have shown that chronic ingestion of small quantities of a central stimulant, methyl phenidate, produces

significant decrements in body weight. These changes in body weight were not capable of being accounted for by reduction in food or water intake. Behavioural studies with the drug have shown that it has marked psychomotor effects (Meier *et al.*, 1954). These effects were, however, obtained with drug doses within the therapeutic range. Rats treated with the dose of methyl phenidate employed in these experiments (200 μg. per ml. of drinking water) did not exhibit any psychomotor effects. It was felt, however, that subtle energy losses may occur during the rat's normally quiescent period, when the animal would not be under observation. To investigate this possibility, rats were placed in the activity cage using the method outlined in the previous paragraph.

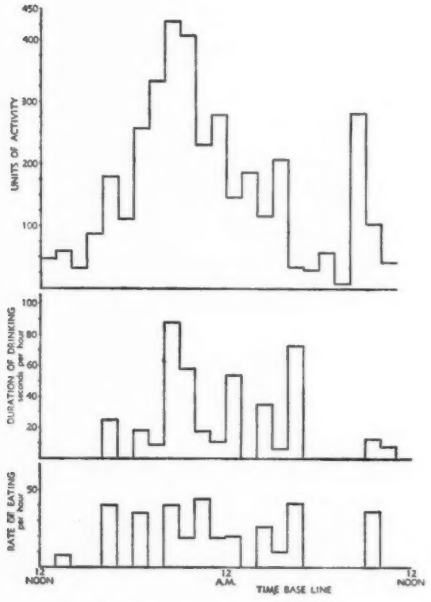

Fig. 7. Control treatment.

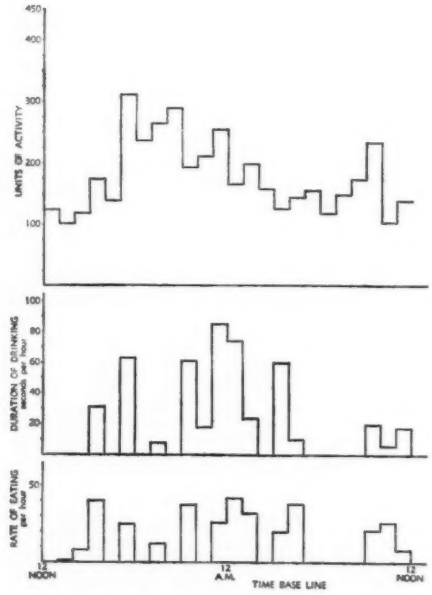

Fig. 8. Experimental treatment, 200 μg. methyl phenidate.

Typical results are shown in Fig. 7. Although these graphs are only the results procured from one animal they do typify the general results obtained from this experiment. It can be seen from the graph that during the hours of 5 a.m. to 4 p.m. not only was the rat inactive, but also very little eating and drinking occurred. The exception was around 9 a.m. when the general stimulus level was probably raised owing to the early morning activity in the College as a whole. By contrast the data obtained from the drugged animal

show that the rat maintained an abnormally raised level of activity during its otherwise quiescent period. It was evident from results such as those described here that methyl phenidate produces prolonged periods of restlessness. The metabolic requirements for such periods of energy expenditure cannot be found to have been supplied by an increased food intake, so that the body weight reserves were called upon which resulted in a growth weight plateau.

REFERENCE.

Meier, R., Gross, F., and Tripod, J. (1954): Klin. Wochenschr., 32, p. 445.